

Docket No.: 27373/33638A
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Weichselbaum et al.

Application No.: 09/964,042

Confirmation No.: 1056

Filed: September 26, 2001

Art Unit: 1635

For: Treatment of Tumors with Genetically
Engineered Herpes Virus

Examiner: J. E. Angell

DECLARATION UNDER 37 C.F.R. § 1.132

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Bernard Roizman, Sc.D., declare that:

2. I am a named co-inventor, along with Ralph Weichselbaum and Richard Whitley, on the above-identified patent application. I am familiar with the contents of the patent application and make this declaration to provide information that may be relevant to examination of the application. A copy of my *curriculum vitae* is attached as Exhibit A.

3. I am aware that the Examiner of the above-identified patent application is maintaining that Advani et al., Int. J. of Radiation Oncology Biology Physics vol. 39(2), Supplement 1, 251 (1997) (*i.e.*, the Advani abstract, a true copy of which is attached as Exhibit B), a reference of record, discloses the administration of herpes simplex virus R7020 to mice bearing human tumor xenografts resulting in tumor reduction. I am further aware that the Examiner has taken the position that Carroll et al., Ann. Surg. 224:323-330 (1996) (*i.e.*, Carroll, a true copy of which is attached as Exhibit C), another reference of record, discloses the use of herpes simplex virus hrR3, an attenuated HSV, to treat colon carcinoma cells that have metastasized to the liver, and asserts that Carroll's use of the attenuated HSV hrR3 to treat a non-CNS tumor provides a reason to adapt the Advani method of treating CNS tumors using HSV R7020 to the treatment of non-CNS tumors, thereby arriving at the subject matter of the pending claims.

4. Neither Advani nor Carroll discloses a method for reducing any tumor mass by administering an attenuated HSV to a patient. Moreover, neither Advani nor Carroll provides any indication that a method of reducing tumor mass with an attenuated HSV could be achieved at a safe, therapeutic dosage.

5. I am a co-author of the Advani abstract, which summarizes an experiment to measure the level of replication of two herpes simplex viruses, i.c., HSV R3616 and HSV R7020. The Advani abstract discloses that a single quantity of virus (2×10^7 pfu) was administered to mice bearing human glioma tumor xenografts. At specified time intervals, tumors were removed, homogenized, and viral titers were determined using conventional techniques and permissive Vero cells. For tumors exposed to HSV R3616, those time intervals were days 3, 5, 7 and 14 post-infection; for HSV R7020, those time intervals were days 3, 5 and 7, but not day 14. The results of determining the viral titers revealed that the concentration of HSV R7020 had dropped to about zero by day 7 and, hence, there was no scientific purpose served by determining viral titers of HSV R7020 beyond this point in time.

6. In the specification of the above-referenced application, Example 1 reveals that an SQ-20b tumor xenograft exposed to HSV R7020 "began to regress 13 days after infection . . ." Specification, page 8, lines 11-13. There is no evidence in the references of record I have reviewed, which includes Advani and Carroll, that would indicate to one of ordinary skill in the art that HSV R7020 would reduce a glioma tumor mass and, in view of the disclosure in the above-captioned specification that HSV R7020 did not begin to reduce an SQ-20b tumor until 13 days post-infection, there would not have been a reasonable scientific basis for inferring tumor mass reduction from the seven-day replication study of HSV R7020 reported in the Advani abstract.

7. Consistent with the foregoing observations, the Advani abstract does not disclose or suggest that HSV R7020 administration to a mouse bearing a human glioma xenograft resulted in reduction of that tumor or any other tumor. The single HSV R7020 experiment described in the Advani abstract was stopped at 7 days post-infection and the only evidence of record relating to HSV R7020-induced tumor mass reduction is the 13 days post-infection required to begin to see SQ-20b tumor mass reduction, as described in the above-captioned application and not in the art of record. Thus, even if the seven-day replication study reported by Advani had assessed tumor mass or reduction thereof, it is unlikely that any mass reduction would have been observed at the seven-day time point. Moreover, the

experiments described in the Advani abstract measured only virus replication in the tumor and virus replication levels alone do not provide a useful measure of either tumor mass reduction or safety of the virus.

8. One of ordinary skill in the art relevant to the pending claims would have a fundamental understanding of oncology and would understand, at a minimum, the basic scientific principles underlying viral oncology. Those principles include a positive correlation between the amount of an oncolytic virus administered to tumor cells and the level of destruction of those tumor cells, the positive, and typically rapid, growth rate of tumor cells, the negative correlation between the amount of an oncolytic virus administered and the safety of such administration to the host organism, and that effective treatment may involve reduction of tumor growth rate, tumor stasis (*i.e.*, neither tumor progression nor regression) or tumor regression (*e.g.*, a reduction of tumor mass). See U.S. Patent No. 5,342,947, col. 14, lines 19-23, attached as Exhibit D, for disclosure relating to tumor stasis.

9. One of ordinary skill in the art, aware of the principles noted in paragraph 8, would understand that a therapeutically effective and safe dose of a particular oncolytic virus is dependent on the virulence of that virus towards tumor cells, the rate of growth of the tumor cells in the host organism being treated, and the virulence of that virus towards healthy cells of the host organism.

10. The Advani abstract does not disclose the dose-response relationship of HSV R7020 administered to glioma tumor cells in mice. The Advani abstract is also silent on the rate of gliomal tumor cell growth in mice, although it was known in the art that glioma tumors grow rapidly, due at least in part to the greater proportion of cells actively growing than is found in healthy tissues. See Schold et al., J. Neuro-Oncology 1:5-14 (1983) attached as Exhibit E.

11. The Advani abstract also lacked any disclosure relating to the safety of HSV R7020 at the administered dosage level, other than noting that this attenuated virus was *less attenuated* than HSV R3616, and thus more virulent than HSV R3616 but less virulent than wild-type HSV. Notably, no healthy mouse tissue was examined in the study reported in the Advani abstract and no assessment of the health of mice injected with R7020 was disclosed in Advani.

12. As established in the preceding paragraphs, the Advani abstract did not expressly disclose that HSV R7020 administration resulted in a reduction in glioma tumor mass. In addition, the Advani abstract did not implicitly or inherently make such a disclosure

because neither the abstract nor the knowledge in the art established that one of ordinary skill in the art would recognize that administration of 2×10^7 pfu HSV R7020 would produce a rate of glioma cell killing that exceeded the rate of glioma cell growth for xenografts in mice having an open-ended size of ">200 mm³," which would be understood to mean greater than 200 mm³.

13. The skilled artisan, having reviewed the Advani abstract, would have had no expectation that HSV R7020 would be capable of reducing tumor mass at a safe dosage. The methods disclosed in Advani may result in reduction of tumor growth rate, in tumor growth stasis, or in tumor mass reduction. Advani only demonstrated that HSV R7020 replicates in glioma cells and, thus, there was no teaching as to the ability of HSV R7020 to reduce tumor mass in a glioma tumor, or any other type of tumor. Moreover, the skilled artisan would have recognized that increasing the dosage of an attenuated HSV composition would not inherently result in tumor mass reduction. In particular, a dose of attenuated HSV required to achieve tumor mass reduction may be unattainable with a virus that is highly attenuated (*i.e.*, viral cell killing does not, at any dosage, exceed tumor growth rate) or, in the case of a more virulent HSV, may result in serious side effects in the patient before a tumor mass-reducing dose is achieved. Likewise, the addition of radiation therapy in combination with an attenuated HSV would not necessarily lead to tumor mass reduction at a safe dosage.

14. Further, a reference in the Advani abstract to "treatment" did not distinguish between treatment resulting in slowed tumor growth or tumor stasis versus treatment resulting in tumor regression. Only the latter form of treatment would result in a reduction of tumor mass.

15. For the foregoing reasons, the Advani abstract does not provide a reasonable basis for one of ordinary skill in the art to believe that any amount of HSV R7020 would be sufficiently effective to kill tumor cells at a rate that exceeded the rate of tumor growth, thereby resulting in a reduction of tumor mass.

16. With respect to the issue of safety, the Advani abstract summarized an experimental determination of a time series of viral titers in homogenized mouse xenografts. Advani's administration of 2×10^7 pfu of HSV R7020 to mice results in inoculation with a greater amount of virus (per unit of subject mass) than had previously been determined to be safe in animal model systems (Meignier et al., J. Infect. Dis., 158:602-614, 1988 (Exhibit F) and Meignier et al., J. Infect. Dis. 162:313-322, 1990 (Exhibit G); references of record). For example, the lethal dose 50 (LD₅₀) for R7020 was previously determined to be approximately

2.7×10^6 when administered to mice intracranially, which is lower than the dose administered in the experiments described in the Advani abstract (*see, e.g.*, Table 1 of Meignier et al., 1988; Exhibit F). Advani, focused on a study of the replication of HSV, did not examine and did not disclose an analysis of any tissue other than the cancerous xenograft. The Advani abstract was silent on any possible effect of HSV R7020 on any healthy tissue, other than to suggest that HSV R7020 would be *less safe* (*i.e.*, less attenuated) than HSV R3616.

17. For reasons elaborated in the preceding paragraphs, one of ordinary skill in the art would not have understood that a tumor mass-reducing dose of HSV R7020 (or any other HSV modified in conformity with the claims pending in the above-captioned patent application) would be safe to administer to a patient based on a review of any of the art being cited against the pending claims, including the Advani abstract. Accordingly, one of ordinary skill in the art did not know, and would not have developed an expectation, that there would be any amount of HSV R7020 that would be a therapeutically effective amount (*i.e.*, a safe and effective amount) to reduce tumor mass because there was no disclosure or suggestion that HSV R7020 would be sufficiently safe to use at an effective dosage level.

18. I am further aware that the Examiner has cited Carroll as disclosing an attenuated HSV being used to treat a non-CNS tumor, thereby assertedly providing motivation to modify Advani's method to arrive at a method of using HSV R7020 to safely reduce the mass of non-CNS tumors.

19. One of ordinary skill in the art would not have been motivated to modify Advani's method based on the disclosure of a non-CNS tumor being exposed to attenuated HSV hrR3 because the mechanism by which HSV hrR3 is attenuated is different from the mechanism by which HSV R7020 is attenuated.

20. HSV R7020 is a multi-mutated HSV having a deletion of one set of inverted repeats and the genes located therein as well as deletions in two other genes. More particularly, HSV R7020 lacks all or portions of the U_L24, and U_L56 genes, and contains only one of the two copies of the α 0, γ 134.5, ORF O, and ORF P genes. In addition, the U_L23 gene was placed under a different promoter in a new location of the HSV genome relative to that of wild-type HSV. Additionally, the HSV-2 genes encoding glycoproteins G, D, I and part of E were inserted in place of the deleted inverted repeat sequences. The HSV hrR3 disclosed in Carroll is an HSV deleted for the U_L39 gene encoding the large subunit of ribonucleotide reductase (ICP6).

21. HSV R7020 is attenuated relative to wild-type HSV. That attenuation is due to the absence of inverted repeats within the HSV R7020 genome, augmented by the absence of the U₁56 gene and of an intact U₁23 gene.

22. HSV hrR3, in contrast to HSV R7020, retains the inverted repeat structure of the wild-type HSV genome. HSV hrR3 is attenuated relative to wild-type HSV because the deletion of U₁39 in HSV hrR3, leading to a failure to express the large subunit of ribonucleotide reductase, renders the virus deficient in synthesizing the nucleotide building blocks it needs for viral replication. Host nucleotides may be available in a given cell, however, and the deletion of U₁39 does not interfere with the ability of the virus to use host nucleotides in replication. The reduced availability of nucleotide building blocks impedes, but does not prevent, successful infection.

23. HSV hrR3 is attenuated through a mechanism completely different from the mechanism responsible for attenuation of HSV R7020. The fact that each of these viruses is less virulent than wild-type HSV (i.e., each is attenuated) does not mean that these two viruses are interchangeable.

24. One of ordinary skill in the art would not be motivated to modify Advani's method to use HSV R7020 to treat non-CNS tumors based on Carroll's disclosure of the use of HSV hrR3 to infect colon carcinoma because the mechanisms of attenuation of these two viruses differ. One of ordinary skill would understand that a demonstration that an HSV (e.g., HSV hrR3) with a particular form of attenuation could successfully infect tumor cells more effectively than healthy cells would not be relevant to whether a different HSV (e.g., HSV R7020), with a different form of attenuation, could be successfully used in analogous methods.

25. For the foregoing reasons, one of ordinary skill in the art would not look to Carroll for guidance in modifying any method disclosed in the Advani abstract because Carroll's HSV hrR3 is attenuated by a mechanism completely unrelated to the mechanism by which Advani's HSV R7020 is attenuated.

26. Moreover, Carroll provided no indication that HSV hrR3, or any other attenuated HSV, could be used in a method of reducing tumor mass.

27. The disclosure of Carroll demonstrated that HSV hrR3 was able to destroy cancer cells *in vitro*, but *in vivo* studies described in Carroll only demonstrated that tumor nodules were *infected* by HSV hrR3. This deficiency was highlighted by the authors

of Carroll in the addendum to the paper discussion section where it is stated “[w]e have only demonstrated in vitro cytotoxicity and in vivo targeting.” See, page 7, paragraph 6 of Carroll. Thus, Carroll provided no guidance as to the ability of HSV hrR3, or any other attenuated HSV, to reduce tumor mass in non-CNS tumors (or any other type of tumor).

28. Carroll also provided no disclosure to suggest that a safe dose of HSV hrR3, or any other attenuated HSV, could be achieved that would also be effective to reduce the mass of a non-CNS tumor. To the contrary, upon reviewing the disclosure of Carroll, a skilled artisan would have questioned the safety of using an attenuated HSV. In particular, *in vivo* studies reported in Carroll disclosed virus-mediated *lacZ* expression (*i.e.*, a marker of viral infection) in “areas of normal liver that had no clear evidence of tumor metastases.” See page 4, paragraph 4 of Carroll. In the paper’s addendum, skilled workers in the field raised concerns regarding the safety of HSV hrR3. For example, Dr. Timothy J. Eberlein questioned whether the detection of virus infection in normal tissue meant that the “treatment is less specific and more toxic?” See page 6, paragraph 8 of Carroll. Likewise, Dr. Jeffery A. Norton suggested that, if examined, there is likely to be “some toxicity, that is infection of other rapidly dividing cells, like bone marrow and intestinal mucosa.” See page 6, paragraph 14 of Carroll. Even the authors of Carroll acknowledged safety concerns with regard to HSV hrR3, stating that “[t]he presence of hrR3 herpes virus in cells other than tumor cells remains a major concern for us.” See page 7, paragraph 1 of Carroll. Thus, the disclosure of Carroll would have suggested to skilled artisans in the field that a tumor mass-reducing dosage of an attenuated HSV such as hrR3 could not be safely achieved.

29. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Feb 14 2009
Date

Bernard Roizman
Bernard Roizman

EXHIBIT A

CURRICULUM VITAE

June, 2008

Personal data:

Name Bernard Roizman

Work address: The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 East 58th Street Chicago Il. 60637.

Home address: 5555 South Everett Ave, Chicago Il 60637

Education:

Temple University, Philadelphia, Pennsylvania 1949-1954, B.A. 1952; M.S. 1954.
The Johns Hopkins University, Baltimore, Maryland 1954-1956; Sc.D. 1956.

Awards and Honors:

Pasteur Award, Illinois Society of Microbiology, 1972; Esther Langer Award, 1974.
Centennial Medal, Institut Pasteur, Paris, France 1987; First annual ICN International Prize in Virology, 1988; Sesquicentennial Gold Medal, University of Ferrara, Italy, 1991; J. Allyn Taylor International Prize in Medicine, 1997; Bristol-Myers Squibb Award for Distinguished Achievement in Infectious Disease Research, 1998; Abbott-ASM Lifetime Achievement Award, 2008.

Elected: Member of the National Academy of Sciences, 1979; Fellow, American Academy of Arts and Sciences, 1991; Fellow, American Academy of Microbiology, 1992; Institute of Medicine, 2001; Fellow, American Association for the Advancement of Science, 2004. Honorary Member, Hungarian Academy of Sciences, 1995, Foreign Member, Chinese Academy of Engineering, 2000.

Honorary degrees: Doctorate in Humane Letters, Governors State University, IL. 1984; Doctorate in Medicine and Surgery. University of Ferrara, Italy 1991; Doctorate in Science, University of Paris, France, 1997, Doctorate in Medicine, Univ. of Valladolid, Spain, 2001; Professor (honoris causa) Shandong Academy of Medical Sciences, China, 1985; Peking Union Medical College, China, 2002, Shandong Univ, 2003; Qingdao Univ. 2003; Jiatong Univ Beijing 2007.

Honorary Fellow, Pan American Cancer Cytology Soc., 1973; Fellow, Japanese Soc. for the Promotion of Science, 1989. Honorary member, Italian Soc. for Virology, 2002.

Outstanding Alumnus in Public Health Award for 1984; Johns Hopkins Univ. Society of Scholars, The Johns Hopkins University, 1988.

Schneierson Visiting Prof., Mount Sinai School of Medicine, New York, N.Y. 1982; Visiting Prof., Univ. of Michigan, 1989; Visiting Prof., Univ. of Bologna, Italy, 1991; Sulkin Visiting Prof. Southwest Med. School 2004; Sackler Fellow, Univ. of Tel Aviv, 1995.

NIH Outstanding Investigator Award 1988-2001; NIH-NCI Merit award, 2003. recipient, Bristol Myers Squibb Unsolicited Research Grant Award in Infectious Diseases, 1991-1995.

Positions Held:

(1). Academic

Medical Institutions, The Johns Hopkins University: Instructor of Microbiology, 1956-1957; Research Associate of Microbiology, 1957-1958; Assistant Prof. of Microbiology, 1958-1965.

Division of Biological Sciences, The University of Chicago:

Associate Professor of Microbiology, 1965-1969; Professor of Microbiology, 1969-1970; Professor of Microbiology and Biophysics, 1970-1981; Joseph Regenstein Professor of Virology in the Departments of Microbiology and Biophysics & Theoretical Biology 1981-1983; Current: **Joseph Regenstein**

Distinguished Service Professor of Virology in the Departments of Molecular Genetics & Cell Biology, Biochemistry & Molecular Biology, and in Microbiology.

Chairman, Interdepartmental Committee on Virology, 1969-1985; 1987-2001

Chairman, Department of Molecular Genetics and Cell Biology, 1985-88.

(2) Editorial

Member, Editorial Board of Journal of Hygiene, 1958-1961.
 Member, Editorial Board of Journal of Infectious Diseases, 1965-1969.
 Member, Editorial Board of Journal of Virology, 1970 –
 Member, Editorial Board of Intervirology, 1972-1985.
 Advisory Editor for Progress in Surface Membrane Science, 1972
 Member, Editorial Board of Archives of Virology, 1975-1981.
 Member, Editorial Board of Virology, 1976-1978; 1983-
 Member, Editorial Board of Microbiologica, 1978-
 Associate Editor, Cell, 1979-1981.
 Member, Editorial Advisory Board, Antiviral Agents Bulletin, 1988-2002
 Member, Editorial Board, Methods in Molecular Biology, 1988-
 Member, Editorial Advisory Board, Encyclopedia of Virology, 1990-
 Editor-in-Chief, Infectious Agents and Disease, 1991-1996
 Member, Editorial Board of Gene Therapy, 1994-
 Member, Editorial Board, J. of Human Virology 1997-2001
 Member, Editorial Board, Encyclopedia of Molecular Medicine, Wiley, 2001
 Member, Editorial Board, J. of Virological Methods 1999-
 Member, Editorial Board of Acta Pathologic, Microbiologica, et Immunologica Scandinavica 2004-
 Member, Board of Editors, Mt. Sinai Journal of Medicine.

(3) Consultantships, Memberships on National Committees, etc.

A) Grant Review Panels

Member, Special virus Cancer Program, Developmental Research Working Group, National Cancer Institute, National Institutes of Health, 1967-1971.
 Consultant, National Cancer Institute, N.I.H. 1967-1973.
 Member, Steering Committee, Human Cell Program, Cell Biology Division, National Science Foundation, 1971-1974.
 Member, the American Cancer Society Advisory Committee on Cell Biol. and Virol., 1970-74.
 Consultant, National Science Foundation, 1972-1974.
 Member, Medical Advisory Board of the Leukemia Research Foundation, 1972-1977.
 Member, Experimental Virology Study Section, Research Grants Review Branch, National Institutes of Health, 1976-1980.

B) International Organizations

Member, Herpesvirus Study Group, International Committee for the Taxonomy of Viruses, 1971- Chairman, 1971 - 1993
 Member, WHO/FOA Herpesvirus Work–ng Team, 1972-1981.
 Member, International Microbial Genetics Commission, International Association of Microbiological Societies, 1979-1986.
 Member, Subcommittee on Vertebrate Viruses, International Committee for the Taxonomy of Viruses, 1981- Chairman, Scientific Advisory Board, Showa University Research Institute for Biomedicine in Florida, 1983- ; Member, 1991- and Chairman, Board of Directors, 1991-1997.

C) National Organizations

Member, Scientific Advisory Council, New York Cancer Institute, 1971-89.
 Member, External Advisory Committee, Emory University Cancer Center, 1973-81
 Member, Board of Scientific Consultants, Sloan Kettering Institute, 1975-81.
 Member, N.I.A.I.D. Task Force on Virology, 1976-1977.
 Member, Board of Trustees, Goodwin Institute for Cancer Research, 1977-

Member, External Advisory Board, Northwestern Univ. Cancer Center, 1979-88.

Member, National Institute of Medicine, Committee for Establishing Vaccine 1983-1985.

Member, Briefing Panel on Prevention and Treatment of Viral Diseases, Office of President's Science Advisor, 1986.

Chairman, Bristol-Myers Squibb Infectious Disease Award Committee, 1991-1995

Member, N.I.A.I.D. Blue Ribbon Panel on Bioterrorism and its implications for Biomedical Research, 2002.

Member, External Scientific Advisory Board, Malaria Research Institute, Johns Hopkins University School of Public Health, 2002-

D. Industrial Organizations

Consultant, Abbott laboratories, 1987-2000;

Avid Tech. 1992-1995; ENI, Italy 1987-1991;

Institut Merieux, Lyon France 1979-1991;

Genta, Inc. 1987-1993;

Lederle Laboratories, 1988-1992;

Schering Plough Research Institute 1988-1997;

Searle 1993-1994;

Syntro Corp. 1981-1986;

Medigene AG (Formerly Neurovir) 1995-

Co-Founder, consultant, member of the Board of Directors - Aviron Inc. 1992-2002.

Consultant Nurel 2004-2005

E) Organization of Scientific Meetings

Convener, *The 1st International Workshop on Herpesvirus's*, Cold Spring Harbor, N.Y., 1972.

Lecturer, American Foundation for Microbiology, 1974-1975.

Vice Chairman, Program Committee 3rd International Congress on Virology, Madrid, Spain, 1975.

Member, International Organizing Committee, Sixth Pan American Cancer Cytology Congress, 1978.

Co-Director, *Seminars on Advances in Cancer Biology* (Cancer Virology), Aspen, Colorado, 1977.

Co-Chairman, *New York Academy of Sciences Conference on Genetic Variation*, York, N.Y., 1979.

Co-Organizer, *International Workshop on Herpesviruses*, Bologna, Italy, 1981.

Co-Organizer, *International Conference on Immunobiology and Prophylaxis of Herpesvirus Infections*, Ft. Lauderdale, Florida, 1983, 1985; Marco's Island 1987; Fukuoka, Japan, 1989; S'. Petersburg, 1991; 1995; Hokkaido, Japan, 1993; Tampa Fl, 1995, Mishima Japan, 1997, Il Ciocco, Pisa, 1999, Osaka Japan, 2001, Taormina Italy 2003, Osaka Japan, 2005, Orvieto Italy, 2007.

Organizer, *Banbury Conference on Viral Latency*; Cold Spring Harbor, 1992.

Co-Organizer, *First Japan Workshop on Herpesviruses*, Osaka Japan, 1992.

Co-Organizer, *IRBM Symposium on Molecular Biology of Viral Latency*, Rome Italy, 1992.

Co-Organizer, *UCLA Symposium on Molecular Biology of Human Viral Pathogens*, 1993.

Organizer, Colloquium on *The effects of changes In human ecology and behavior on human infectious diseases*. National Academy of Sciences, September 1993.

Co-Organizer, Symposium on *Current Frontiers in Virology*, Chicago Il, May 1994.

Co-Organizer, *International Workshop on Herpesviruses*, DeKalb, Il, July, 1996.

Co-Organizer, National Academy of Sciences colloquium on *Genetic Engineering of Viruses and of Virus Vectors*, Irvine, CA, June 1996

Co-organizer, *Keystone Symposium on Molecular Approaches to Human Viral Vaccines*. Snowbird, Utah, 1999.

Fellowships:

Lederle Medical Faculty Award, 1960-1961; Scholar in Cancer Research of the American Cancer Society at

Institut Pasteur (with Andre Lwoff) Paris, France, 1961-1962; U.S.P.H.S. Career Development Award, 1963-1965; Faculty Research Associate, American Cancer Society, 1966-1971; Travelling Fellow, International Agency for Research Against Cancer (with Dr. George Klein, Karolinska Institutet, Stockholm, Sweden), 1970

Memberships in Scientific Societies:

American Association of Immunologists, American Society for Microbiology, American Society for Biochemistry and Molecular Biology, British Society for General Microbiology, American Society for Virology, International Society for Antiviral Research.

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1. Robinson, L.B., Wichelhausen, R.H. and Roizman, B. Contamination of human cell cultures by pleuropneumonia-like organisms. *Science* **124**: 1147-1148, 1956.
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3. Chanock, R., Roizman, B. and Myers, R. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterization. *Am. J. Hyg.* **66**: 281-290, 1957.
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5. Roizman, B., Hopken, W. and Mayer, M.M. Immunochemical studies of poliovirus. II. Kinetics of the formation of infectious and non infectious type I poliovirus in three cell strains of human derivation. *J. Immunol.* **80**:386-395, 1958.
6. Roizman, B., Mayer, M.M. and Rapp, H.J. Immunochemical studies of poliovirus. III. Further studies on the immunologic and physical properties of poliovirus particles produced in tissue culture. *J. Immunol.* **81**:419-425, 1958.
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9. Hoggan, M.D. and Roizman, B. The effect of temperature of incubation on the formation and release of herpes simplex virus in infected FL cells. *Virology* **8**:508-524, 1959.
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22. Roizman, B. and Roane, P.R., Jr. A physical difference between two strains of herpes simplex virus apparent on sedimentation in cesium chloride. *Virology* **15**:75-79, 1961.
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2. B. Roizman (Editor): **Herpesviruses**, Vol. 1, Plenum Press, New York, 1982.
3. B. Roizman (Editor): **Herpesviruses**, Vol. 2, Plenum Press, New York, 1983.
4. B. Roizman (Editor): **Herpesviruses**, Vol. 3, Plenum Press, New York, 1985.
5. B. Roizman and C. Lopez (Editors): **Herpesviruses**, Vol. 4, Plenum Press, New York, 1985.
6. B.N. Fields, D.M. Knipe, R.M. Chanock, J.L. Melnick, B. Roizman and R.E. Shope (Editors): **Virology**, Raven Press, New York, 1985.
7. B.N. Fields, D.M. Knipe, R.M. Chanock, J.L. Melnick, B. Roizman and R.E. Shope (Editors): **Fundamental Virology**, Raven Press, New York, 1986. Translated into Russian, MIR Publishers, Moscow, 1989.
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13. B. Roizman, Ed. Proceedings of the colloquium on **Changes in human ecology and behavior: Effects on infectious diseases.** Proc. Nat Acad. Sci. USA **91** 2377-2468, 1994.
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New Vaccine Development. Establishing of Priorities: Volume II. Diseases of importance in the Developing Countries. National Academy Press, Washington, D.C., 1986

Former Trainees and Associates

Name	Training Period	Degree	Graduate Year	Institution	Present position
Predoctoral					
Laure Aurelian	1962-65	Ph.D.	1966	Johns Hopkins	Prof. Depart. of Pharm., Univ. Maryland Med. School,
Baltimore Md					
Steven Bachenheimer	1967-72	Ph.D.	1972	U. of Chicago	Prof. Depart. of Microbiol. and Immunol. Univ. N.
Carolina, Chapel Hill, N.C.					
William Batterson	1974-84	Ph.D.	1981	U. Of Chicago	Microbiol. U. Texas-Houston
Daniel Braun	1981-83	Ph.D.	1983	U. Of Chicago	Staff Physician, Elli Lily and Co. Indianapolis IN.
				M.D. 1979 U. Michigan	
Timothy Buchman	1975-78	Ph.D.	1978	U. of Chicago	Prof. Surg., Dir. Critical Care & Burn unit. Wash. Univ.
St Louis.				M.D. 1980 U. of Chivago	
Kara Carter	1990-96	Ph.D.	1996	U. of Chicago	Associate Director, Genzyme Corp.
Chang, Y-E	1991-96	Ph.D.	1996	U. of Chicago	Lawyer
Ana Chee	1997-03	Ph.D.	2003	U. of Chicago	Research Associate Northwestern Univ. Med. School.
Joanie Chou	1983-86	Ph.D.	1986	U. of Chicago	President, Akure Inc. Chicago IL.
Lizette Durand	2002-07	Ph.D.	2007	U. of Chicago	Research Associate, Univ of Chicago
Randall Fawl	1987-93	Ph.D.	1993	U. of Chicago	Wistar Inst., Univ of Penna.
Niza Frenkel	1969-72	Ph.D.	1972	U. of Chicago	Prof. University of Tel Aviv, Israel
Deidre Furlong	1978-81	Ph.D.	1975	U. of Chicago	Research Assoc. Harvard Med. School, Boston, Mass.
Veronica Galvan	1994-99	Ph.D.	1999	U. Chicago	Postdoc. Buck Center for Aging, CA
Wade Gibson	1967-72	Ph.D.	1973	U. of Chicago	Professor, Dept. of Pharm. Johns Hopkins Med. School.
Baltimore Md.					
Ryan Hagglund	1999-03	Ph.D.	2003	U. of Chicago	UC Law School
David Hoggan	1956-59	Sc.D.	1959	Johns Hopkins	Staff Scientist, NIAID
Jeff Hubenthal-Voss	1983-88	Ph.D.	1988	U. Washington	Senior Scientist, BASF Bioresearch, Cambridge, MA.
Paola Jones	1972-79	Ph.D.	1979	U. of Chicago	Research Assoc. Ben May Institute, Univ. of Chicago
Elliott Kieff	1966-71	Ph.D.	1971	U. of Chicago	Prof., Dep. of Medicine and Microb. & Mol. Gen.,
Harvard Med. School				M.D. 1966	J. Hopkins
Thomas Kristie	1981-86	Ph.D.	1986	U. of Chicago	Research Scientist (tenured) NIAID
Michael Lagunoff	1989-95	Ph.D.	1995	U. of Chicago	Assoc. Prof. University of Washington (WA)
Yu Liang	1999-05	Ph.D.	2005	U. Chicago.	Post Doctoral Trained, NIH.
Fenyong Liu	1988-92	Ph.D.	1993	U. of Chicago	Prof. UC Berkeley
Richard Longnecker	1982-88	Ph.D.	1987	U. of Chicago	Prof. Microbiology Northwestern Med. School. Chicago
Il.					
Susan Mackem	1976-82	Ph.D.	1982	U. of Chicago	Senior Clinical Investigator, Laboratory of

Pathology, NCI,	M.D. 1984 Johns Hopkins			
Nancy Michael	1986-93	Ph.D.	1993 U. of Chicago	Res. Associate, University of Chicago (Deceased).
Louise McCormick	1989-96	Ph.D.	1996 U. of Chicago	Res. Assoc. Asist Prof. Emory Med. School
Vincent Morris	1966-69	Ph.D.	1969 U. of Chicago	Prof., Univ. W. Ontario, Canada
Lawrence Morse	1975-77	Ph.D.	1978 U. of Chicago	Prof. of Ophthalmology, U.C. California, Davis.
	M.D. 1982 UCLA			
Joashua Munger	1996-01	Ph.D.	2001 U.of Chicago	Assistant Professor, Univ. of Rochester
William Ogle	1992-98	Ph.D.	1998 U. of Chicago	Assist. Prof. University of Florida. Gainsville.
Phillip Pellett	1981-85	Ph.D.	1985 U. of Chicago	Prof.. Wayne State Med. School
Kim Poffenberger	1979-84	Ph.D.	1984 U. of Chicago	Staff Scientist, Fed. Drug Administration
Glenn Randall	1993-99	Ph.D.	1999 U. of Chicago	Assist. Professor, University of Chicago.
Charles Van Sant	1994-00	Ph.D.	2000 U. of Chicago	Scientist, Abbott Laboreatories
Amy E. Sears	1978-85	Ph.D.	1985 U. of Chicago	Assoc. Member, Tampa Bay Rsearch Inst. St Petersburg.
FL				
Jerome Schwartz	1966-69	Ph.D.	1969 U. of Chicago	Vice Pres/Med Direct., Applied Clinical
Communications, Inc.			Parsippany, NJ.	
Sandra Silver	1980-85	Ph.D.	1985 U. of Chicago	Senior Scientist, Repligen Corp.
Benjamin Smith-Donald	2001-06	Ph.D.	2006 U. of Chicago	MSTP Program
David, Spector,	1988-91	Ph.D.	1991 U. of Chicago	Oncology practice, Quad city Iowa
			M.D., 1993 U. of Chicago.	
Patricia G. Spear	1965-69	Ph.D.	1969 U. of Chicago	Prof. & Former Chair, Dept of Microbiol., Northwestern
Med. School, Chicago				
Susan Spring	1964-69	Ph.D.	1969 U. of Chicago	Program Director, NCI
Samuel Wadsworth	1970-74	Ph.D.	1974 U. of Chicago	Vice President, Molecular Biology, Genzyme Corp.

Postdoctoral

Mathias Ackermann	1983-85	DVM	1982 U. Zurich,	Professor, Swiss Inst. Virol. Zurich Switzerland
Minas Arsenakis	1985-87	Sc.D.	1985 LaTrobe U.	Professor, Dept. of Biology, U. of Thessaloniki, Greece
		Australia		
Joel Baines,	1989-93	Ph.D.	1989 Cornell Univ.	Professor, Microbiol., Cornel. Univ. Ithaca N.Y
			DVM	
John Blaho	1989-94	Ph.D.	1989 U. of Alabama	Head, Virology Division, Medican Diagnostic Lab. Hamilton NJ.
Renato Brandimarti	1993-99	Ph.D.	1993 U. Bologna	Assist. Professor, Univ, of Bologna Italy.
Renato Bruni	1993-98	Ph.D.	1993 U. of Zurich, Sw.	Scientist, Tribeca, N.Y.
Kevin Cassady	1995-98	M.D.		Assist Prof. of Pediatrics, Univ of Ala. Birmingham
Enzo Cassai	1971-72	Ph.D.	1970 U. of Ferrara	Prof. Microbiol. Univ. of Ferrara, Italy
Anthony Conley	1977-80	Ph.D.	1977 Michigan State	Senior Scientist, Merck, PA

Esclatine, Audrey	2000-03	Ph.D.	Univ of Paris,	Assist Prof. Univ of Paris.
Fineschi, Beatrice.	1997-01	Ph.D.	1997 Univ. of Chicago	Instructor, Univ. of Chicago.
Gary Hayward	1974-76	Ph.D.	1972 Auckland, NZ	Prof. Pharmacol, Med. School. Johns Hopkins
Bin He	1994-98	Ph.D.	1993 Purdue Univ.	Assoc. Prof. Univ. of Il Med. School Chicago IL
Carolyn Herz	1981-82	Ph.D.	1981 Cornell U.	Res. Assoc. Walther Oncology Center, Ind.
Robert Honess .	1972-75	Ph.D.	1972 Birmingham,	Head, Div. Virol. NIMR, MRC, London, UK (Deceased)
Kazuhiko Igarashi	1991-93	Ph.D.	1991 Tohoku Univ. M.D.	Prof. Depart. of Biochem. Univ. of Hiroshima School of Med. Japan
Yasushi Kawaguchi	1995-97	Ph.D	1994 U. of Tokyo DVM	Assoc. Prof. Tokyo University, Japan
Robert Jacob	1976-79	Ph.D	1975 Syracuse U.	Assoc. Prof. Microbiol., Univ. of Kentucky Med. School, Lexington, KY.
Bernard Jacquemont	1972-74	Ph.D.	1971 U. Claude	Sr. Sci. INSERM, Lyon, Bernard-Lyon France
Frank J. Jenkins	1984-87	Ph.D.	1984 Penn State	Assoc. Prof. Univ of Pittsburg Med.
John Keller	1968-70	Ph.D.	1966 M.I.T.	Prof. Biochemistry Univ. of Health Sci., North Chicago Il.
Robert King	1990-92	Ph.D.	1990 Purdue Univ.	Research Director, Dow Chemical.
Marilyn Kozak	1972-74	Ph.D.	1972 Johns Hopkins	Prof. Biochem., Rutgers University
David Knipe	1976-79	Ph.D.	1976 M.I.T.	Prof. Microbiology & Mol. Genetics, Harvard Med. Sch.
Rosario Leopardi	1994-98	Ph.D.	1994 Univ. of Turku Finland P.D. 1990 Catania Italy	Assist. Prof. Karolinska Univ. Sweden
Sandra LeMaster	1979-80	Ph.D.	1978 U. of Penn.	Research Assoc. Microbiology, U. of Cincinnati
Markovitz, Nancy	1994-00	Ph.D.	1994 U. of Texas Austin Staff Scientist FDA	
P. Mavromara-Nazos	1979-84	Ph.D.	1984 U. of Michigan	Senior Member, Pasteur Inst., Athens, Greece
Robert Millette	1973-75	Ph.D.	1964 Cal. Tech	Prof. of Biology, U. of Oregon
Edward Mocarski	1979-83	Ph.D.	1979 U. of Iowa	Professor, Emory Univ Atlanta GA.
Terese Ng	1991-97	Ph.D	1991 U. of Iowa	Res. Scientist, Abbott Labs
Leonore Pereira	1974-77	Ph.D.	1974 U. of Frankfort	Prof. Univ of California San Francisco.
Sofia Perazzo	1997-99	M.D.	1997 U. of Buenos Aires Resident, U. of Buenos Aires	
Leonard Post	1979-81	Ph.D.	1979 U. of Wisc.	Vice President for Research, Onyx Inc. California.
Frances C. Purves	1988-93	Ph.D.	1986 U. of Glasgow,	Res. Scientist Tularek Inc. S. San Francisco, CA.
Roller J. Roller	1989-94	Ph.D.	1989 Harvard	Prof., Med. Microbiology, Univ. of Iowa.
M. G. Romanelli	1988-90	Ph.D.	1985 U. of Ferrara	Assoc. Prof. of Genetics, University of Verona. Italy
William Ruyechan	1976-78	Ph.D.	1975 U. Ill-Urbana	Prof. Biochem., University of N.Y. at Buffalo
Sciortino, M.T.	1997-01	Ph.D.	1997 U. of Messina	Assist. Prof. U. of Messina, Italy.
Saul Silverstein	1971-74	Ph.D.	1971 U. of Florida	Prof. & Chair, Microbiol., P&S, Columbia Univ.
Mikiko Suzuki	2000-04	DDS	1994 Tokyo Med Univ.	Assist. Prof. Toyama Med. & Pharm Univ. Toyama Japan
		Ph.D.	1998 Tokyo Med Univ.	
Robert Sydiskis	1965-67	Ph.D.	1965 Northwestern	Prof. of Microbiology, Univ. Maryland Med. School.
Enrique Tabares	1977	Ph.D.	1972 U. of Madrid	Prof. Microbiology, Med. Sch. U. Autonoma, Madrid

Mauro Tognon	1977-80	Ph.D.	1975 U. of Ferrara	Prof. Applied Biol. Univ. of Ferrara, Italy
Trgovcic Joanne.	1998-02	Ph.D.	1994 U. N. Carolina	Assist. Prof. of Pathology, Ohio State Univ.
Edward K. Wagner	1967-70	Ph.D.	1967 M.I.T.	Prof. of Molecular Biology, UC – Irvine (Deceased)
Patricia L. Ward	1985-99	Ph.D.	1985 U. of Chicago	Staff member, Museum of Science and Industry, Chicago
Kent Wilcox	1975-79	Ph.D.	1974 Johns Hopkins	Assoc. Prof. of Microbiology, Univ. Wisc. Milwaukee
Hanns Wolf	1975-77	Ph.D.	1974 U. Erlangen,	Prof. and Dir. Inst. for Med. Microbiology, Regensburg Univ., Germany
Manfred Wolff	1973-76	Ph.D.	1973 U. Bonn, WG	Prof. Inst. fur Virologie and Microbiologie, Universitat Witten/Herdecke
Guo-Jie Ye Gainesville FL.	1996-01	Ph.D.	1994 China	Associate Director, Applied Genetic Technologies Corp.

Visiting Scientists (Past)**Michael Fenwick, Ph.D. Sabbatical Reader, Univ. Oxford, England****G. Campadelli-Fiume, Ph.D. Sabbatical Prof. of Virol. Univ. of Bologna Italy****Ian Halliburton, Ph.D. Sabbatical Reader, Department of Microbiology, University of Leeds, UK****Tao Hung M.D. Sabbatical Professor, Chinese Academy of Preventive Medicine, Beijing.****Veijo Hukkanen Sabbatical Univ of Turkku, Finland****Alex Kohn, Ph.D. Sabbatical Professor, Univ. of Tel-Aviv Med. School****Augustine Hajime Koyama, D.Sc. Sabbatical Prof. of Virology, Wakayama Medical University, Wakayama, Japan****Bodil Norrild, Ph.D. Sabbatical Associate. Prof. of Microbiology Univ of Copenhagen Denmark****Filatov, Felix, Ph.D. Visiting Scientist; Member, Ivanovsky Institute for Virology, Russian (USSR) Academy of Sciences, Moscow.****Benito Reguero M.D. Sabbatical Professor and Chairman, Department of Microbiology, the University of Santiago de Compostela, Spain.** (Sabbatical leave 1/93 to 12/93).**Markert, James M.D. Visiting Assist Prof. of Neurosurgery, Univ of Alabama 9/95-4/96****Allessandra Stefan, Sc.D. Visting Scientist, University of Bologna, Italy; Fall 1997.****Yugi Isegawa M.D. Sabbatical Assist. Prof. Microbiology, Univ of Osaka Japan, Jam-Nov 1997****Wang, Janwei, Visiting Scientist, Associate Professor, Institute for Virology Beijing 8-11/2002****Special Fellows:****Bradford S. McGwire Howard Hughes Medical Student Fellow 1989-1990**

EXHIBIT B

2021

ENHANCED REPLICATION OF ATTENUATED HSV-1 IN IRRADIATED HUMAN GLIOMA XENOGRAFTS

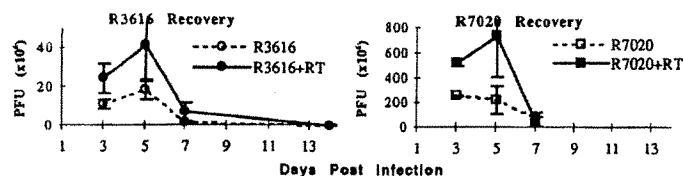
Sunil J. Advani¹, Yasushi Kataoka¹, Greg S. Sibley¹, Paul Y. Song¹, Dennis E. Hallahan¹, Bernard Roizman², and Ralph R. Weichselbaum¹

¹The Department of Radiation and Cellular Oncology and ²The Marjorie B. Kovler Viral Oncology Laboratories
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Purpose: Previously we had shown that combining ionizing radiation (IR) with attenuated replication competent HSV-1 (R3616) significantly increased glioma xenograft eradication compared to IR or virus alone. One hypothesis is that IR induces cell factors that contribute to augment viral replication thereby increasing the efficacy of attenuated HSV-1. The purpose of this study was to examine if IR altered viral replication of attenuated HSV-1 in glioma xenografts.

Material and Methods: Human U-87MG glioma cells were grown in the hindlimb of athymic mice and grown to >200 mm². Tumors were infected with 2x10⁷ plaque forming units (pfu) of R3616 (34.5') or R7020 (multimutated, 34.5') on day 0 and irradiated with 20 Gy on day 1 and 25 Gy on day 2. Tumors were harvested 3, 5, 7, and 14 days after viral injection. Tumors were homogenized and sonicated. Serial dilutions of tumor extract were overlaid on Vero cells to determine the number of pfu. In addition, in-situ hybridization to HSV-1 DNA was performed on tumors harvested at day 7.

Results: In-situ hybridization revealed larger numbers of glial cells infected with HSV along with a greater distribution in the irradiated tumors compared to non-irradiated tumors. We next quantified viral particles in infected tumors +/- IR:



Conclusion: Herein we demonstrate radiation enhanced viral replication as one of the interactive effects of combining IR and attenuated HSV in treating glioma xenografts and a potential therapeutic motif in the treatment of gliomas. To reduce normal tissue toxicity of HSV in glioma therapy, viruses must be attenuated. However, attenuating the virus compromises its replication and thus its potential efficacy. Our results indicate that IR augments the amount of virus recovered from human glioma xenografts for up to 3 days post IR. The results do not appear to be related to a specific mutation in the herpes genome but rather to herpes viruses in general. Yields of R7020 were greater than R3616 since R7020 is less attenuated than R3616. IR provides a conducive local environment for viral replication and can specifically target enhanced viral replication to the irradiated tumor bed. We hypothesize ionizing radiation induces cellular gene(s) involved in glioma DNA repair that interact in the viral replication cycle to enhance viral replication. This effect is transient in that as the levels of the cellular induced gene(s) fall viral replication is no longer enhanced, and attenuated viruses diminish the threat of systemic toxicities.

2022

MOLECULAR REQUIREMENTS FOR RADIATION-ACTIVATED RECOMBINATION

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Purpose/Objective: The major stumbling block to successful gene therapy today is poor gene transfer. We hypothesized that ionizing radiation might activate cellular recombination, and so improve stable gene transfer. We further hypothesized that known DNA-damage-repair proteins might also be important in radiation-activated recombination.

Materials and Methods: The effect of irradiation on stable gene transfer efficiency was determined in human (A549 and 39F) and rodent (NIH/3T3) cell lines. Continuous low dose rate and multiple radiation fractions were also tested. Nuclear extracts were made and the effect of irradiation on inter-plasmid recombination/ligation determined. Multiple DNA damage-repair deficient cell lines were tested for radiation-activated recombination.

Results: A significant radiation dose-dependent improvement in stable plasmid transfection (by as much as 1300 fold) is demonstrated in neoplastic and primary cells. An improvement in transient plasmid transfection is also seen, with as much as 85% of cells transiently expressing b-galactosidase (20-50 fold improvement). Stable transfection is only improved for linearized or nicked plasmids. Cells have improved gene transfer for at least 96 hours after irradiation. Both fractionated and continuous low dose rate irradiation are effective at improving stable gene transfer in mammalian cells, thus making relatively high radiation dose delivery clinically feasible. Inter-plasmid recombination is radiation dose dependent in nuclear extract assays, and the type of overhang (3', 5' or blunt end) significantly affects recombination efficiency and the type of product. The most common end-joining activity involves filling-in of the overhang followed by blunt end ligation.

Adenovirus is a linear, double stranded DNA virus. We demonstrate that adenoviral infection efficiency is increased by irradiation. The duration of transgene expression is lengthened because the virus integrates with high efficiency (~10% of treated cells) into cellular DNA.

The mechanism of radiation enhanced stable gene transfer requires effector proteins to accomplish the recombination. The Ku proteins, which are required for V(D)J recombination, account for at least 90% of radiation induced recombination. There is also an absolute requirement for the Ataxia Telangiectasia gene (ATM) for any radiation induced recombination to occur, although the transfection efficiency in unirradiated cells is unaffected by ATM. Removal of p53 by transfection of E6 (Human Papilloma Virus) significantly inhibits radiation activated recombination, and this is confirmed in nuclear extract recombination assays.

Conclusions: Ionizing radiation activates a recombination pathway which may be useful in gene therapy. The molecular mechanism of radiation activated recombination requires a number of DNA-damage-repair proteins.

EXHIBIT C

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Enhancement of Gene Therapy Specificity for Diffuse Colon Carcinoma Liver Metastases with Recombinant Herpes Simplex Virus [Scientific Paper]

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Abstract TOP

Objective: The authors determined whether a recombinant herpes simplex virus (HSV) vector could destroy human colon carcinoma cells *in vitro* and whether the vector would selectively replicate in colon carcinoma liver metastases but not surrounding hepatocytes *in vivo*.

Background: The HSV vector hrR3 is defective in the gene encoding ribonucleotide reductase and contains the lacZ reporter gene. Ribonucleotide reductase is expressed in actively dividing cells and generates deoxyribonucleotides for DNA synthesis. hrR3 replicates only in actively dividing cells that can provide ribonucleotide reductase in complementation, but not in quiescent cells such as normal hepatocytes.

Methods: hrR3-mediated lysis of HT29 human colon carcinoma cells was first determined *in vitro*. For *in vivo* studies, athymic BALB/c nude mice underwent intrasplenic injection of HT29 and intrasplenic injection of hrR3 7 days later, and were killed 7 days after viral injection. Their livers were examined histochemically for lacZ expression.

Results: All the HT29 cells were destroyed *in vitro* when hrR3 was added at a titer of 1 plaque-forming unit per 10 tumor cells. One hundred one of 105 tumor nodules examined in liver sections from mice treated by intrasplenic injection of hrR3 demonstrated lacZ expression. Minimal betagalactosidase activity was present in normal liver.

Conclusions: The hrR3 HSV vector effectively destroys HT29 human colon carcinoma cells at very low multiplicities of infection. Differential expression of ribonucleotide reductase between liver metastases and normal liver allows hrR3 to selectively replicate in tumor cells with minimal replication in surrounding normal liver. Further investigation of HSV-based vectors as oncolytic agents for liver metastases is warranted.

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This year, colorectal carcinoma will develop in approximately 135,000 people in the United States. Although mortality rates from this disease are declining, more than 55,000 people are expected to die of colorectal carcinoma in 1996.¹ Liver metastases remain the most prevalent form of metastatic disease, and it is estimated that liver metastases will develop in 75% of patients who die of colorectal carcinoma.² Although a small proportion of patients with colorectal carcinoma liver metastases may benefit from surgical resection, most patients have multiple metastases and are not candidates for surgical resection. Patients with multiple metastases have been treated with a variety of modalities, including intravenous chemotherapy, regional chemotherapy, cryosurgery, and chemoembolization. However, these approaches remain palliative; rarely-if ever-are patients with diffuse metastases cured by these treatments.²

Several gene therapy strategies have been examined for their therapeutic potential in the treatment of cancer. These approaches have been based on 1) introduction of genes that produce multiple copies of RNA decoys; 2) expression of transdominant proteins that can functionally replace mutant or deleted cellular proteins; 3) modification of adoptively transferred T cells; 4) expression of cell surface antigens to enhance the antitumor response; 5) expression of ribozymes that cleave specific DNA sequences; 6) intracellular production of antibodies to bind to specific proteins; 7) expression of foreign enzymes that render cells susceptible to otherwise nontoxic prodrugs; 8) production of intracellular toxins that lead to cell death; 9) modification of hematopoietic stem cells to decrease toxicity from chemotherapy; or 10) infection with oncolytic viruses that can themselves destroy tumor cells.³

Numerous vehicles for delivery of genes to both normal cells and cancer cells have been developed, including retrovirus, adenovirus, vaccinia virus, adeno-associated virus, and herpes virus.⁴ Most of the previously described gene therapy approaches for cancer require direct intratumoral injection of these vectors.⁵⁻⁷ This strategy is not feasible for patients with *diffuse* liver metastases. Hurford and colleagues treated diffuse hepatic micrometastases in mice, created by splenic injection of sarcoma and breast carcinoma cell lines, with intrasplenic injection of a retroviral producer cell line. They demonstrated selective gene transfer to tumor deposits. There are several drawbacks to retroviral vectors, including the theoretical capability of causing neoplastic transformation of normal cells by insertional mutagenesis. Accordingly, we have investigated a strategy using herpes simplex virus (HSV) type 1 vectors for treatment of *diffuse* liver metastases.

Herpes simplex virus has been explored as a vehicle for gene transfer into the central nervous system, but studies using HSV have demonstrated cytotoxicity from cellular lysis.^{9,10} These apparently negative characteristics of HSV vectors can be adapted for therapeutic purposes in the treatment of cancer. Entry of wild-type HSV into cells leads to a sequential cascade of viral gene expression that ultimately results in the production of multiple progeny virions and cell death.¹¹ Herpes simplex virus vectors demonstrate significant oncolytic activity and reporter gene transfer in experimental brain tumor models.¹²

The normal liver is similar to the brain because it has minimal mitotic activity.¹³ In contrast, liver metastases demonstrate significantly greater mitotic activity. The activity of enzymes necessary for DNA replication, such as ribonucleotide reductase, is increased in tumors compared with normal tissues.¹⁴ Accordingly, one strategy to develop HSV vectors that selectively lyse diffuse liver metastases rather than normal hepatic parenchymal cells involves deletion of specific genes necessary for viral replication, such as ribonucleotide reductase. Such vectors would only be able to replicate in actively dividing tissue that could provide ribonucleotide reductase in complementation.^{15,16}

In the current study, we have assessed the feasibility of employing a mutant HSV vector in the treatment of diffuse colon carcinoma liver metastases. We have examined paired patient specimens representing normal liver and colon carcinoma liver metastases for ribonucleotide reductase expression, and confirmed that expression is virtually undetectable in normal liver. In contrast, extremely high levels of ribonucleotide reductase were found in colon carcinoma metastases. Then we examined the ability of the ribonucleotide reductase deficient HSV vector (hrR3) to infect and destroy HT29 human colon carcinoma cells *in vitro*. hrR3 destroyed HT29 cells at titers of only 1 plaque-forming unit per 10 tumor cells. Finally, we injected hrR3 intrasplenically into nude mice bearing diffuse HT29 liver metastases. hrR3 specifically and efficiently targeted diffuse liver metastases. Negligible hrR3 infection of normal liver was identified.

MATERIALS AND METHODS TOP

Cell Lines, Tumor Specimens, Antibodies, and Viral Vectors TOP

The human colon carcinoma cell line HT29 was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium with Hamm's F12 supplement and 8% (v/v) fetal calf serum. Human tissue specimens were immediately frozen in liquid nitrogen in the operating room and stored until further processing.

The monoclonal antibody MAS 378 AD203 (Accurate Chemical and Scientific Corp., Westbury, NY) recognizes the M1 subunit of ribonucleotide reductase. The monoclonal antibody A-5441 (Sigma Chemical Co., St. Louis, MO) recognizes beta-actin.

The hrR3 HSV vector was obtained from S. Weller (Connecticut Medical School, University of Connecticut Health Center, Farmington, CT) and is defective in ribonucleotide reductase expression.^{15,16} This vector contains the *Escherichia coli* lacZ gene inserted into the ribonucleotide reductase gene locus. The lacZ gene is driven by the ICP6 immediate-early gene promoter. hrR3 was passaged on parental African Green Monkey (Vero) cells and stored in stocks at -80°C before use.

Western Blot TOP

For Western blot analysis, tumor tissue was homogenized in 50 mmol/L Tris, pH = 8; 150 mmol/L sodium chloride; 0.2% sodium azide, 100 µg/mL phenylmethylsulfonyl fluoride; 1 mg/mL aprotinin; and 1% Triton X-100. Total protein concentration was measured using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL). Lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, and transferred to nitrocellulose filters by electroblotting at 4°C. After blocking for 1 hour in phosphate-buffered saline (PBS) containing 5% dry milk, the filters were incubated with MAS 378; washed in PBS containing 1% dry milk and 0.2% Tween-20; incubated with horseradish peroxidase-conjugated antimouse antibody (Amersham Corp., Arlington Heights, IL); washed in 150 mmol/L sodium chloride, 10 mmol/L Tris, pH = 8, 0.05% Tween-20; and specific proteins were detected using an enhanced chemiluminescence system (Amersham Corp.). Filters were reprobed with A-5441 to detect beta-actin.

In Vitro Cell Infection Assay TOP

HT29 cells were plated in 96 well plates at a concentration of 5000 cells per well and allowed to grow for 48 hours. The media were removed and the cells were washed with serum-free media. Then hrR3 virus in 50 µL of serum-free media was added to the cells in multiplicities of infection (number of plaque-forming units per cell) ranging from 0.0001 to 1. The cells were gently shaken every 15 minutes for 45 minutes at 37°C, and 50 µL Dulbecco's modified Eagle's medium-F12 supplemented with penicillin, streptomycin, and 16% fetal calf serum was then added after this initial viral adsorption period. Six days later, the media were replaced with RPMI 1640 without phenol red containing 0.5 mg/mL thiazolyl blue (MTT; Sigma Chemical Co.) for 1 hour at 37°C.¹⁷ The media were removed, and formazan crystals were solubilized with 50 µL dimethyl sulfoxide (DMSO). After the plate was vigorously shaken, the optical density of each well was measured using an automatic plate reader (Anthos HT2, Anthos Labtec Instruments, Salzburg, Austria) with a 550-nm measurement wavelength and a 650-nm reference wavelength. The percentage cell survival was determined by calculating the ratio of OD_{550/650} of hrR3-infected cells to the OD_{550/650} of mock infected cells. All experiments were performed in quadruplicate.

Hepatic Metastasis Model and Treatment with Herpes Virus Vector TOP

Animal studies were performed in accordance with guidelines issued by the Massachusetts General Hospital Subcommittee on Research Animal Care. Pathogen-free, 4- to 5-week old male athymic BALB/c nude mice were allowed to acclimate for 1 week. Cells were detached from plates in 5 mmol/L edetic acid in PBS and resuspended in Hank's balanced salt solution, free of calcium and magnesium; 5 × 10⁶ cells in a single-cell suspension were injected intrasplenically, as described.¹⁸ One week later, either 1 × 10⁷ plaque-forming units of hrR3 in 100 µL of PBS or 100 µL of PBS alone were injected into the spleen. One week later, the mice were killed and their livers were examined for lacZ expression.

Liver Sections and Histochemical Staining TOP

Livers were snap frozen in liquid nitrogen, and frozen sections were prepared. Sections were fixed in 4% paraformaldehyde in 10 mmol/L sodium phosphate and 0.9% sodium chloride (pH = 7.3) and then washed with PBS. Slides were incubated for 48 hours at 37°C in a solution containing 35 mmol/L potassium ferricyanide, 35 mmol/L potassium ferrocyanide, 2 mmol/L magnesium chloride, 0.01% sodium deoxycholate, 0.02% Nonidet P40, and 0.2% of a solution containing 40 mg 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal; Sigma Chemical Co.) in 1 mL of dimethylformamide at pH = 7.3. The slides were rinsed with water and counterstained with cresyl violet.

RESULTS TOP

Ribonucleotide Reductase Expression in Human Colon Carcinoma Metastases and Normal Liver TOP

We examined ribonucleotide reductase expression in biopsies of colon carcinoma liver metastases and adjacent normal liver from patients by Western blot analysis. As expected, ribonucleotide reductase expression in the relatively quiescent liver was extremely low compared with the high levels detected in the colon carcinoma metastases (Fig. 1). These data supported the feasibility of selectively targeting liver metastases with an engineered herpes viral vector defective in ribonucleotide reductase that should replicate only liver metastases where ribonucleotide reductase is available in complementation.

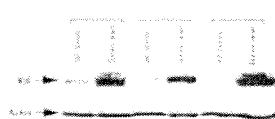


Figure 1. Expression of ribonucleotide reductase M1 subunit (RR) in paired specimens representing human colon carcinoma liver metastases (liver met) and adjacent normal liver (nl liver) from three patients measured by Western blot analysis. Equivalent amounts of protein were loaded in each lane as demonstrated by beta actin expression.

Herpes Simplex Virus Vector-Mediated Lysis of HT29 Colon Carcinoma Cells *In Vitro* TOP

On the basis of the aforementioned findings, we sought to examine the ability of a ribonucleotide reductase defective HSV vector to infect and destroy human colon carcinoma cells *in vitro*. The hrR3 HSV vector is defective in the large subunit of the viral ribonucleotide reductase because of an insertional mutation. This vector has the *E. coli* lacZ gene driven by the ICP6 immediate early promoter inserted into the ribonucleotide reductase gene locus. We wanted to examine the ability of this HSV vector to destroy tumor cells in culture, regardless of the pH and nutrient changes associated with prolonged incubation. Accordingly, we chose 6 days as an

endpoint for the assay, and first determined conditions that would allow log phase growth of the HT29 cells for 6 days after initial seeding.

Six days after infecting HT29 human colon carcinoma cells with hrR3 at multiplicity of infection (MOI) values ranging from 0.0001 to 1.0, we quantitated the number of viable cells using the colorimetric MTT assay. We also quantitated the number of viable uninfected control cells. hrR3 destroyed approximately 70% of the HT29 cells infected at a titer of 1 plaque-forming unit per 100 tumor cells (multiplicity of infection = 0.01; Fig. 2). Virtually 100% of the cells were destroyed after 6 days when hrR3 was added a titer of 1 viral particle per 10 tumor cells (multiplicity of infection = 0.1). Cytopathic effects were observed in culture as early as 24 hours after infection. These data indicate that hrR3 is an extremely effective cytopathic agent against HT29 cells *in vitro*.

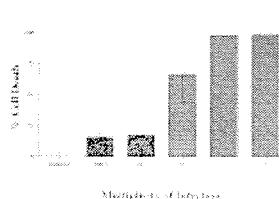


Figure 2. hrR3-mediated lysis of HT29 colon carcinoma cells *in vitro*. The percentage of cells destroyed 6 days after infection with hrR3 was quantitated by the MTT assay. Data are presented as the mean \pm standard deviation of quadruplicate experiments.

We also examined the ability of the ICP6 promoter to drive expression of the lacZ gene in the HT29 cells. HT29 cells were fixed in glutaraldehyde 72 hours after hrR3 infection and stained with X-gal. Strong lacZ expression was detected (data not shown), indicating that we would be able to identify hrR3 infected cells *in vivo* by staining for lacZ expression.

Splenic Injection of Herpes Virus Vector to Infect Liver Metastases TOP

On the basis of having demonstrated differential expression of ribonucleotide reductase in normal liver compared with metastases and highly effective hrR3-mediated destruction of colon carcinoma cells *in vitro*, we next sought to determine if hrR3 would selectively replicate in diffuse liver metastases *in vivo* when injected into the portal vein. To produce diffuse liver metastases, 5×10^6 HT29 cells were injected into the spleens of nine nude mice. Seven days later, 1×10^7 plaque-forming units of hrR3 were injected intrasplenically. The animals were killed 7 days later, and liver sections were examined histochemically for lacZ gene expression. One hundred five tumor nodules were examined in several liver sections from all nine mice, and lacZ gene expression was detected in 101 (96%) of these nodules. The extent and distribution of lacZ gene expression were relatively similar for all the tumor nodules (Fig. 3). Five to 25% of HT29 colon carcinoma cells stained positive for lacZ expression in most of the tumor nodules. We also detected a minimal amount of beta-galactosidase activity in areas of normal liver that had no clear evidence of tumor metastases. In a separate set of experiments, mice bearing liver metastases introduced via the spleen were injected with PBS only (no hrR3 virus). Tumor nodules examined in several liver sections demonstrated no evidence of lacZ expression. No beta-galactosidase activity was noted in normal liver in these animals.



Figure 3. Histochemical detection of lacZ expression in HT29 liver metastases 7 days after splenic injection of hrR3. (A) Tumor nodule examined at 100 \times magnification. (B) Tumor nodule examined at 200 \times magnification. Sections were counterstained with cresyl violet.

DISCUSSION TOP

Our initial interest in HSV vectors for gene delivery and destruction of liver metastases arose from experimental results using HSV vectors to treat brain tumors.^{19,20} The ribonucleotide reductase deficient hrR3 vector can mediate complete tumor regression and 20% long-term survival in rats harboring an intracerebral 9L neoplasm. Furthermore, negligible hrR3 infection of surrounding normal astrocytes and endothelial cells occurs. This therapeutically favorable differential susceptibility of malignant *versus* normal brain tissue to cytolytic infection by hrR3 presumably results from the extremely high levels of mitotic activity within tumors compared with the surrounding normal brain tissue.¹²

We have examined the feasibility of this strategy to treat liver metastases. We assumed that ribonucleotide reductase levels in normal liver would be much lower than in colorectal carcinoma liver metastases. We were unable to locate any reports describing ribonucleotide reductase levels in colorectal carcinoma. Accordingly, we examined several pairs of normal liver and liver metastases specimens by Western blot analysis and confirmed our assumption. Although the difference in expression was quite dramatic, very low levels of ribonucleotide reductase expression were detected in normal liver. Further analysis by immunohistochemistry is necessary to identify the source of this expression.

Several features of hrR3 render it an advantageous vector for tumor gene therapy. First, hrR3 is replication conditional and will multiply in dividing cells, such as metastases, and not multiply in nondividing cells, such as hepatocytes.¹⁹ This relative selectivity results from tumor upregulation of ribonucleotide reductase, thereby allowing complementation for hrR3 replication. Second, in addition to its inherent cytopathic effect, hrR3 may be used to deliver cytotoxic genes to tumor cells. For example, hrR3 possesses an

endogenous thymidine kinase gene that confers chemosensitivity to ganciclovir.¹² The HSV genome also can accommodate exogenous genes. It is estimated that up to 30 kb of the HSV genome can be replaced by exogenous sequences.²¹ Third, hrR3 provides these functions at very low multiplicities of infection.

Direct intratumoral inoculation of hrR3, as was performed for treatment of experimental brain tumors, is not a feasible strategy to treat diffuse liver metastases. Accordingly, we examined the efficacy of intraportal delivery of hrR3 via splenic injection. The efficacy and specificity of lacZ gene expression in tumor cells were striking. Ninety-six percent of histochemically examined tumor nodules stained positive for lacZ expression. The ratio of lacZ expression in tumor nodules was extremely high compared with normal hepatic parenchyma. However, some beta-galactosidase activity was detected in liver sections in which no HT29 tumor cells could be identified by routine cresyl violet counterstaining. There are several potential explanations for this finding. Low levels of ribonucleotide reductase expression in endothelial cells and hepatocytes may have been sufficient to allow complementation for hrR3 replication. Alternatively, insufficient levels of ribonucleotide reductase in these cells may have resulted in aborted replication after lacZ gene expression.¹⁹ Some of the positive staining in normal liver also could be a result of hrR3-infected isolated HT29 cells scattered throughout the liver. It is unlikely that much of the staining in normal liver was due to endogenous beta-galactosidase activity. We carefully controlled the pH of the staining solutions to suppress endogenous beta-galactosidase activity; endogenous mammalian beta-galactosidase is active only in acidic conditions.²² Additional studies are necessary to examine each of these possible explanations for the low level staining present in normal liver.

hrR3 gene expression, as assessed by lacZ expression, was confined to no more than 25% of tumor cells within any tumor nodule. Most often, 5% to 10% of the tumor cells expressed lacZ. LacZ expression was assessed only at 7 days postinfection. Further studies are warranted to assess the level of lacZ expression at various time intervals postinfection to gain insight into the percentage of tumor that ultimately becomes infected with hrR3.

It is unlikely that infection of every tumor cell in a liver could be achieved by portal injection of hrR3, and a few surviving cells may be sufficient to re-establish a tumor. However, this problem may be surmountable. Numerous studies demonstrate that viral gene expression is required in only a portion of a tumor for complete tumor regression.²³ The death of uninfected tumor cells adjacent to transduced cells has been termed the bystander effect.²⁴ This effect may be exploited to enhance tumor destruction. As previously noted, hrR3 possesses a viral thymidine kinase gene that converts ganciclovir to a toxic metabolite. Ganciclovir treatment has been shown to potentiate the antitumor effect of hrR3 in the treatment of gliosarcomas in a rat model.¹² Although the mechanisms involved in this potentiation are not elucidated fully, the bystander effect probably is an important component of the enhanced tumor regression. Assessment of the effects of ganciclovir on hrR3-infected colorectal carcinoma is necessary for further development of therapeutic strategies.

Thus, differential expression of ribonucleotide reductase between liver metastases and normal liver allows hrR3 to selectively replicate in tumor cells with minimal replication in surrounding normal liver. Intraportal injection of hrR3 results in expression of the *E. coli* lacZ gene in more than 95% of tumor nodules. These results warrant further investigation into HSV vector-based therapies against liver metastases.

Acknowledgment TOP

The authors thank Maureen Chase for her help with herpes viral production and measurement of viral titers.

Paper Discussion TOP

DR. SAMUEL A. WELLS, JR. (St. Louis, Missouri): The basic premise of this paper is that gene modification of the herpes virus, by interrupting the ribonucleotide reductase locus, causes selective infection in tumor tissue. This can only be proven by comparing *in vivo* infection of this modified virus to the relative infectivity of genetically unmodified wild-type herpes virus for normal murine liver cells and HT-29 tumor cells, and it seems that this should have been the control.

Some wild-type viruses have been shown to specifically infect and persist in tumor tissue but not normal tissue after administration. Thus, the gene modification may have no relevance to the HT-29 tumor specificity of the virus. Furthermore, viruses typically have a tropism for specific tissue and in liver cells, may not support replication of the herpes virus as well as human tumor cells. This species difference alone may explain the observed results. Data regarding this argument were not presented, and I hope that the authors will be able to clarify my confusion.

DR. WILLIAM C. WOOD (Atlanta, Georgia): I would also like to congratulate Dr. Tanabe and his two investigators on their results. This is a fascinating probe that they have. Ninety-six percent of the nodules had some lacZ expression, but only 5% to 25% of the tumor cells had evidence of infectivity with this agent, often only 5% to 10%.

My first question to Dr. Tanabe is, is that sufficient to eradicate tumor from direct cytotoxicity or from bystander cytotoxicity? What cytology did they find at 7 days? It did not seem striking to the untrained eye on the biopsy just shown.

My second question is, did you inject this virus into the spleens of normal mice to see if there are normal areas in the liver or elsewhere in the body that would take up this virus and be infected with herpes simplex virus (HSV)? To ask the same question in a

different way, do you have any phase 1 toxicity data on the injection of this agent in the murine system?

Third, do you have any phase 2 data from this system? Did these tumor nodules that took up lacZ and appeared to be infected with your HSV variant show a complete response rate or a partial response rate that would enable us to have any evidence as to the direct cytotoxicity of this agent?

I would congratulate you for opening an approach to viral targeting that takes advantage of this potential differential.

DR. TIMOTHY J. EBERLEIN (Boston, Massachusetts): Dr. Tanabe and his colleagues present a potentially useful strategy for the treatment of colorectal liver metastases. Although surgical excision of colorectal metastases offers a 25% to 30% long-term survival rate, the majority of these patients have recurrence. Therefore, a potentially useful treatment that would treat an entire liver with potential micrometastases is desired.

Within this background, Dr. Tanabe takes advantage of the fact the normal liver had minimal mitotic activity. In marked contrast, the tumor in the liver had very high mitotic activity and therefore would utilize the enzymes necessary for DNA replication. One of these enzymes, ribonucleotide reductase, has been shown to be increased in tumors.

The central observation in the study is that by utilizing Western blot analysis, Dr. Tanabe has shown us that normal liver specimens have virtually undetectable ribonucleotide reductase expression yet there is an extremely high level in colon carcinoma liver metastases. It is this differential that forms the basis of a potential treatment utilizing this strategy.

My first question is, what is the expression of the ribonucleotide reductase in other more actively dividing tissues such as epithelial cells in the gastrointestinal tract? If this strategy were to be combined with a liver resection and regenerated liver then ensued, what is the level of ribonucleotide reductase expression in regenerating liver?

This type of treatment strategy has been utilized, as mentioned, for experimental brain tumors. In that model, direct intratumoral inoculation is utilized. This is not feasible for the treatment of diffuse liver metastases.

Dr. Tanabe has shown the efficiency and specificity of tumor cell infection using lacZ gene expression. This is striking. However, there is some beta galactosidase activity detected in normal liver tissue yet no tumor cells are identified by routine counterstaining. Does this imply that retreatment with the herpes simplex virus (HSV) vector will increase beta galactosidase activity in normal liver, thereby making this treatment less specific and potentially more toxic?

In a related issue, although Dr. Tanabe has shown elegant uptake in tumor tissue of this HSV vector, how much *in vivo* tumor cytotoxicity is identified? Did the cytotoxicity actually correlate with gene update? And what might the effect of tumor necrosis, as is often seen in liver metastases, be on the treatment strategy?

Although 96% of the tumor nodules were identified by lacZ gene expression, is there an explanation for why the other nodules were not identified? Similarly, because only 5% to 25% of each tumor nodule stained positive, do you have an explanation for the lack of staining of the remaining tumor nodules?

Finally, I would encourage further studies, as was proposed in the discussion in the manuscript, on the herpes viral thymidine kinase strategy because this model may be an ideal model to study the bystander effect, which has been based on an *in vitro* assay and the mechanism of which has not been satisfactorily worked out in an *in vivo* model.

DR. JEFFREY A. NORTON (St. Louis, Missouri): I would like to rise to say that I enjoyed this paper very much. In our laboratory, we have done similar work with vaccinia virus. We have deleted the ribonucleotide reductase gene, the thymidine kinase gene and the hemagglutinin gene in that virus and had similar results; *i.e.*, relative specificity to the tumor. But I think that the word relative is important in analyzing these data. I would like to ask a few questions, many of which have been already described by the other discussants.

What transgenes do you plan to add to the herpes simplex virus?

The current strategy relies primarily on infection of almost 100% of the tumor cells. The specificity, that is the infection rate of tumor cells *versus* normal liver tissue, is not that great. Therefore, I expect that you will see some toxicity, that is infection of other rapidly dividing cells, like the bone marrow and intestinal mucosa.

Therefore, how do you plan to improve the specificity to see more infection of the tumor cells and less infection of normal tissue?

DR. KENNETH K. TANABE (Closing Discussion): I would again like to give credit to others in my research group: Drs. Carroll, Takahashi, and Chiocca.

The presence of hrR3 herpes virus in cells other than tumor cells remains a major concern for us. Liver cell populations other than hepatocytes, such as Kupffer's cells and endothelial cells, have higher replicative activity than hepatocytes. It is certainly possible that these cells account for the ribonucleotide reductase that we were able to detect in normal liver. Immunohistochemical studies are necessary to localize the source. I suspect that hrR3 can replicate in these normal cells. Furthermore, even in the absence of ribonucleotide reductase in a cell, hrR3 replication may occur, as Dr. Norton points out, albeit at significantly reduced efficiencies. Herpes can also exist in a latent state, unlike many other viruses, a state that we would not be able to detect by examination for lacZ expression. In brief, even though hrR3 is a replication conditional mutant, it is easily conceivable that it could have infected nontumor cells in the liver.

Although an intraportal injection principally targets the liver, hrR3 introduced by this route probably also makes its way into the systemic circulation. Additionally, shed virus from hrR3 that is replicating in the liver tumors may also reach the systemic circulation. Presumably, the organs at highest risk from this systemic spread are those with the highest replicative activity that can provide ribonucleotide reductase in complementation, such as hair follicles, bone marrow, and gut mucosa. As each of the discussants have pointed out, clearly we need to look for systemic hrR3 spread in our animal models. We have already embarked upon these studies with polymerase chain reaction analysis and histochemical staining, but unfortunately I do not have any data to show you today. The hrR3 construct retains an intact thymidine kinase gene which renders the virus susceptible to acyclovir or ganciclovir. We need to carefully document any systemic presence of hrR3 after intraportal injection, as well as its response to acyclovir.

There are several potential explanations for the low level of beta-galactosidase activity that we observed in normal liver. As I mentioned, some cells in normal liver expressed ribonucleotide reductase and hrR3 may have been replicating in these cells. Alternatively, some of this staining detected in what was apparently normal liver may have represented hrR3 replication in diffuse individual scattered HT29 cells that were not detected by routine staining. Some of the lacZ staining may have represented quiescent cells infected with hrR3, in which replication was aborted due to the absence of ribonucleotide reductase and lacZ expression preceded abortion of the replicative process. Lastly, mammalian cells have low levels of endogenous beta-galactosidase activity that could have accounted for some of the blue staining. However, this endogenous mammalian beta-galactosidase activity is most active at acidic pH, whereas *Escherichia coli* beta-galactosidase is most active at neutral pH. We carefully controlled the pH of the staining solutions to minimize this type of background staining. We are currently trying to address each of the possible explanations for the staining seen in normal liver.

Some of the treated tumor nodules did not have any detectable lacZ expression. All of the animals in these experiments were sacrificed at a single time point after injection of hrR3. We really do not understand the temporal sequence of events *in vivo* yet. It may be that some tumor nodules expressed lacZ at day 3 but not at day 7.

For the nodules that did express lacZ at day 7 we do not know the duration of expression. Studies designed to understand the timing of hrR3 gene expression are obviously necessary. For example, we need to know when thymidine kinase expression is maximal to determine when to introduce the pro-drug, ganciclovir.

The limited time permits me to address a couple of the other questions only in brief. We have not demonstrated *in vivo* cytotoxicity in this study. We have only demonstrated *in vitro* cytotoxicity and *in vivo* targeting. We would first like to incorporate into our animal model the use of the pro-drug ganciclovir which gets converted by thymidine kinase into a toxic metabolite.

Dr. Norton asked which transgene we plan to introduce. Herpes does carry its own thymidine kinase gene, and we will make use of it. We are also actively pursuing the introduction of cytosine deaminase as a transgene. As you can see, most of our efforts are centered around the pro-drug approach. The bystander effect seen with pro-drug strategies may yield an effective therapy with transduction of less than 100% of the tumor cells. Dr. Wells brought up an excellent point in his comments. We need to use wild-type herpes simplex virus as a control to confirm our hypothesis. Some work has been done using wild-type herpes simplex virus in the treatment of brain tumors, and wild-type herpes infects with much less specificity in that particular animal model. However, we need to examine this in our model as well.

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Utilizing Tumor Hypoxia to Enhance Oncolytic Viral Therapy in Colorectal Metastases.

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[Abstract] [Fulltext]

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EXHIBIT D



US005342947A

United States Patent [19]

Lackey et al.

[11] Patent Number: 5,342,947

[45] Date of Patent: Aug. 30, 1994

[54] PREPARATION OF WATER SOLUBLE CAMPTOTHECIN DERIVATIVES

[75] Inventors: Karen Lackey, Durham; Daniel D. Sternbach, Chapel Hill, both of N.C.
 [73] Assignee: Glaxo Inc., Research Triangle Park, N.C.

[21] Appl. No.: 960,498

[22] Filed: Oct. 9, 1992

[51] Int. Cl. 5 C07D 491/147

[52] U.S. Cl. 546/41; 546/48;
544/60; 544/125; 544/361; 540/470; 540/481;

540/575; 540/597

[58] Field of Search 546/41, 48; 514/279,
514/283; 562/586; 564/209

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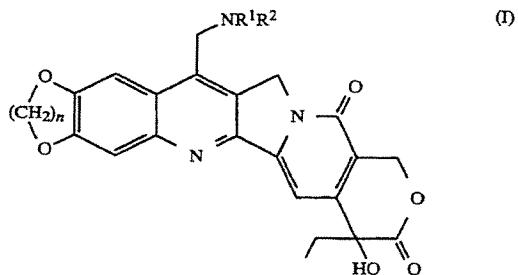
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Primary Examiner—Donald G. Daus
 Attorney, Agent, or Firm—Charles T. Joyner

[57] ABSTRACT

The present invention relates to the synthesis of water soluble, camptothecin derivatives of formula (I),



wherein:

n represents the integer 1 or 2;

R¹ represents independently, hydrogen, lower alkyl, (C₃₋₇)cycloalkyl, (C₃₋₇)cycloalkyl lower alkyl, lower alkenyl, hydroxy lower alkyl, lower alkoxy lower alkyl; and

R² represents hydrogen and

the pharmaceutically acceptable salts thereof.

5 Claims, No Drawings

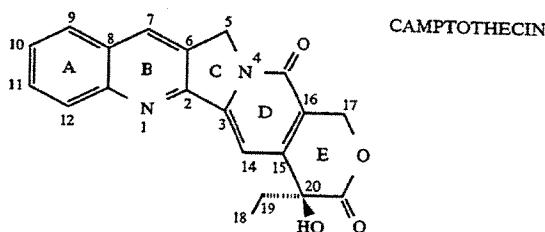
PREPARATION OF WATER SOLUBLE CAMPTOTHECIN DERIVATIVES

The present invention relates to water soluble, camptothecin derivatives substituted in the 7 position, their use in the treatment of tumors and methods of their preparation.

BACKGROUND OF THE INVENTION

Camptothecin, a natural, cytotoxic alkaloid, is a topoisomerase I inhibitor and potent antitumor agent. It was first isolated from the leaves and bark of the Chinese plant, *Camptotheca acuminata*, by Wall, et al. (*J. Am. Chem. Soc.*, 88 3888 (1966)).

As depicted, camptothecin is a fused ring system, composed of a quinoline (A and B), fused to a pyrrolidine ring (C), fused to an alpha-pyridone ring (D) which in turn is fused to a lactone ring (E).



It has an asymmetric carbon at the 20 position making two enantiomeric forms possible. However, the natural occurring compound is found in the "S" configuration as shown above.

Cytotoxic agents are often employed to control or eradicate tumors i.e., they are chemotherapeutic agents. Camptothecin's cytotoxic activity is thought to be directly related to camptothecin's potency as a topoisomerase inhibitor. [For detailed explanations of the topoisomerase function see A. Lehninger, *Principles of Biochemistry*, 813, Worth Publishers, New York (1982); L. F. Liu, "DNA Topoisomerases," *CRC Critical Review in Biochemistry*, 1-24, 15 (1983) and H Vosberg, "DNA Topoisomerases: Enzymes that Control DNA Conformation," *Current Topics in Microbiology and Immunology*, 19, Springer-Verlag, Berlin (1985).] In particular, camptothecin has been shown to be effective in the treatment of leukemia (L-1210) and certain solid tumors in laboratory animals, e.g., see *Chem. Rev.* 23, 385 (1973) and *Cancer Treat. Rep.* 60, 1007 (1967).

Unfortunately, in the clinic camptothecin's promise as an effective antitumor agent has not been completely fulfilled. Camptothecin is essentially insoluble in physiologically compatible, aqueous media, and must be modified to make it sufficiently soluble for parenteral administration, a preferred mode for antitumor treatment. It can be made soluble by forming its sodium salt, that is, by opening the lactone with sodium hydroxide (see F. M. Muggia, et al., *Cancer Chemotherapy Reports*, pt. 1, 56, No.4, 515 (1972)). However, M. C. Wani, et al., *J. Med. Chem.* 23, 554 (1980), reported that the alpha-hydroxy lactone moiety of ring E is an absolute requirement for antitumor activity.

In the art there are examples of modifications and derivatives of camptothecin prepared to improve its solubility in water. Although many of these derivatives were active in *in vitro* and in early animal studies using leukemia (L-1210) models, they were disappointing in

chronic, animal models involving implanted solid tumors.

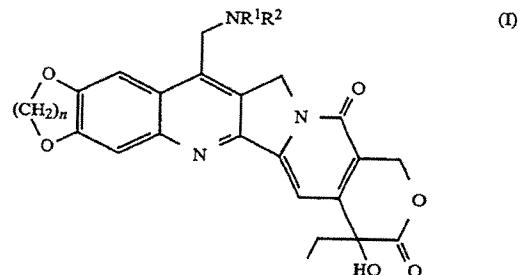
Miyasaka, et al., U.S. Pat. No. 4,399,282, discloses a group of camptothecin derivatives substituted at the 7 position with, inter alia, hydroxymethyl and alkoxymethyl. Further, Miyasaka, et al. in U.S. Pat. No. 4,399,276 discloses camptothecin-7-aldehyde and certain related aldehyde derivatives such as acetals, oximes and hyrazones. More recently, Vishnuvajjala, et al., in U.S. Pat. No. 4,943,579, claimed a series of water-soluble camptothecin derivatives with substituents on the A ring as does Boehm, et al., European Patent application 0 321 122 A2. Other examples of derivatives of camptothecin include Miyasaka, et al., U.S. Pat. No. 4,473,692 and U.S. Pat. No. 4,545,880; and W. Kingsbury, et al., *J. Med. Chem.*, 34, 98 (1991). None of these references reported compounds with antitumor activity greater than that of camptothecin itself.

Wani and co-workers reported that 10, 11-methylenedioxycamptothecin is more potent than unsubstituted camptothecin (see M. C. Wani, et al., *J. Med. Chem.*, 29, 2358 (1986) and 30, 2317 (1987)). However, its water solubility is as poor as camptothecin which seriously limits its clinical utility.

We have now found water-soluble analogs of camptothecin with good, topoisomerase I inhibitory activity *in vitro*, and impressive, antitumor activity *in vivo*.

SUMMARY OF THE INVENTION

One aspect of the present invention are the water-soluble camptothecin analogs of formula (I),



wherein:

n represents the integer 1 or 2; and

- i) R¹ and R² represent independently, hydrogen, lower alkyl, (C₃₋₇)cycloalkyl, (C₃₋₇)cycloalkyl lower alkyl, lower alkenyl, hydroxy lower alkyl, lower alkoxy lower alkyl; or
- ii) R¹ represents hydrogen, lower alkyl, (C₃₋₇)cycloalkyl, (C₃₋₇)cycloalkyl lower alkyl, lower alkenyl, hydroxy lower alkyl or lower alkoxy lower alkyl, and R² represents —COR³, wherein: R³ represents hydrogen, lower alkyl, perhalo-lower alkyl, (C₃₋₇)cycloalkyl, (C₃₋₇)cycloalkyl lower alkyl, lower alkenyl, hydroxy lower alkyl, lower alkoxy, lower alkoxy lower alkyl; or
- iii) R¹ and R² taken together with the linking nitrogen form a saturated 3 to 7 atom heterocyclic group of formula (IA)



wherein: Y represents O, S, SO, SO₂, CH₂ or NR⁴
 wherein: R⁴ represents hydrogen, lower alkyl, perhalo lower alkyl, aryl, aryl substituted with one or more lower alkyl, lower alkoxy, halogen, nitro, amino, lower alkyl amino, perhalo-lower alkyl, hydroxy lower alkyl, lower alkoxy lower alkyl groups or; —COR⁵, wherein: R⁵ represents hydrogen, lower alkyl, perhalo-lower alkyl, lower alkoxy, aryl, aryl substituted with one or more lower alkyl, perhalo-lower alkyl, hydroxy lower alkyl, lower alkoxy lower alkyl groups or;
 the pharmaceutically acceptable salts thereof.

Pharmaceutically acceptable salts include, but are not limited to salts with inorganic acids such hydrochloride, sulfate, phosphate, diphosphate, hydrobromide and nitrate or salts with an organic acid such as acetate, malate, maleate, fumarate, tartrate, succinate, citrate, lactate, methanesulfonate, p-toluenesulfonate, palmitate, salicylate and stearate. Other acids such as oxalic, while not in themselves pharmaceutically acceptable, may be useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable salts.

The lactone ring, ring E, may be opened by alkali metal or alkaline-earth metal bases, for example sodium hydroxide or calcium hydroxide, to form alkali metal or alkaline-earth metal salts of the corresponding open E ring form of the compounds of formula (I). Because of its better solubility in water, the open E ring form may advantageously be purified by conventional recrystallization techniques. Accordingly, said open E ring form may then be used as an intermediate to form the compounds of formula (I), for example by treatment with acid, e.g., hydrochloric acid, and thereby produce a purified form of the compounds of formula (I).

As noted above, the camptothecin moiety has an asymmetric carbon atom at the 20 position making two enantiomeric forms, i.e., "R" and "S" configurations, possible. This invention includes both enantiomeric forms and any combinations of these forms. For simplicity, where no specific configuration at the 20 position is depicted in the structural formulas, it is to be understood that both enantiomeric forms and mixtures thereof are represented. Unless noted otherwise, the nomenclature convention, "(R,S)", denotes a racemic (approximately equal portion) mixture of the R and S enantiomers while "(R)" and "(S)" denote essential optically pure R and S enantiomers respectively. Also included in the invention are other forms of the compound of formula (I), such as solvates, hydrates, polymorphs and the like.

Another aspect of the invention is a method of inhibiting topoisomerase Type I in mammalian cells comprising administering to a patient a topoisomerase inhibiting amount of a compound of formula (I), and a method of treating a tumor in a mammal comprising administering to a mammal bearing a tumor, an effective antitumor

amount of a compound of formula (I). A further aspect comprises pharmaceutical formulations containing a compound of formula (I) as an active ingredient. Methods of preparation of the compounds of formula (I) and the associated novel chemical intermediates used in the synthesis, as taught herein, are also within the scope of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Compounds

As used herein the term "lower" in reference to alkyl and alkoxy means 1-6 carbons and in reference to alkenyl means 3-6 carbons (provided that the double bond is not attached to the carbon which is attached to the nitrogen). The term "perhalo" means all hydrogens have been replaced with a halogen, for example, perhalo lower-alkyl, e.g., trifluoromethyl. The term "aryl" means phenyl or naphthyl.

One group of compounds according to the invention are the compounds of formula (I) wherein:

n represents the integer 1 or 2; and

- i) R¹ and R² represent independently, hydrogen, lower alkyl (e.g. methyl, ethyl) or hydroxy lower alkyl (e.g. hydroxyethyl);
- ii) R¹ represents hydrogen and R² represents —COR³, wherein R³ represents perhalo-lower alkyl (e.g. trifluoromethyl); or
- iii) R¹ and R² taken together with the linking nitrogen form azetidine, pyrrolidine, piperidine, morpholine, thiomorpholine or piperazine (optionally N-substituted with lower alkyl (e.g. methyl), phenyl, phenyl substituted with one or more perhalo-lower alkyl (e.g. trifluoromethyl) or lower alkoxy (e.g. methoxy)) or —COR⁵, wherein R⁵ represents lower alkyl (e.g. butoxy); and

the pharmaceutically acceptable salts thereof.

A sub group of compounds of formula (I) are those compounds wherein:

R¹ and R² represent: independently, hydrogen, lower alkyl, (C₃₋₇) cycloalkyl, (C₃₋₇)cycloalkyl lower alkyl, lower alkenyl, hydroxy lower alkyl, lower alkoxy lower alkyl, and Y represents O, S, CH₂, NH or N(lower alkyl).

Particular compounds of the above sub group are those wherein:

R¹ and R² represent: independently; hydrogen, (C₁₋₄)alkyl, (C₃₋₇)cycloalkyl, (C₃₋₇)cycloalkyl (C₁₋₄)alkyl, (C₃₋₄)alkenyl, hydroxy (C₁₋₄)alkyl, (C₁₋₄)alkoxy (C₁₋₄)alkyl, or taken together with the nitrogen form aziridine, azetidine, pyrrolidine, piperidine, hexamethylenimine, imidazolidine, pyrazolidine, isoxazolidine, piperazine, N-methylpiperazine, homopiperazine, N-methylhomopiperazine, thiazolidine, isothiazolidine, morpholine or thiomorpholine.

Specific compounds of formula (I) are:

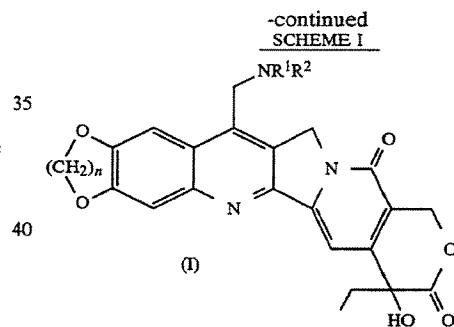
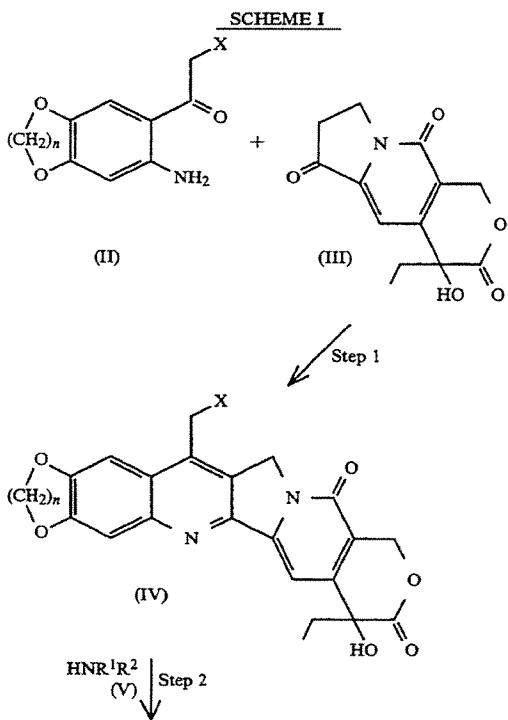
Example Number	Compound Name
1.	7-Dimethylaminomethylene-10, 11-methylenedioxy-20(R,S)-camptothecin,
2.	7-Dimethylaminomethylene-10, 11-methylenedioxy-20(S)-camptothecin,
3.	7-Dimethylaminomethylene-10, 11-ethylenedioxy-20(R,S)-camptothecin,
4.	7-Dimethylaminomethylene-10, 11-ethylenedioxy-20(S)-camptothecin,
5.	7-Morpholinomethylene-10, 11-ethylenedioxy-20(R,S)-camptothecin,
6.	7-Morpholinomethylene-10, 11-ethylenedioxy-20(S)-camptothecin,
7.	7-Pyrrolidinomethylene-10, 11-ethylenedioxy-20(R,S)-camptothecin,
8.	7-Piperidinomethylene-10, 11-methylenedioxy-20(R,S)-camptothecin,
9.	7-Piperidinomethylene-10, 11-ethylenedioxy-20(R,S)-camptothecin,
10.	7-(4-Methylpiperazinomethylene)-10, 11-ethylenedioxy-20(R,S)-

-continued

Example Number	Compound Name
	camptothecin,
11.	7-(4-Methylpiperazinomethylene)-10, 11-ethylenedioxy-20(S)-camptothecin,
12.	7-Diethylaminomethylene-10, 11-methylenedioxy-20(S)-camptothecin,
13.	7-Diethylaminomethylene-10, 11-ethylenedioxy-20(R,S)-camptothecin,
14.	7-Diethylaminomethylene-10, 11-ethylenedioxy-20(S)-camptothecin,
15.	7-N-Methylethanolaminomethylene-10, 11-methylenedioxy-20(R,S)-camptothecin,
16.	7-N-Methylethanolaminomethylene-10, 11-ethylenedioxy-20(RS)-camptothecin,
17.	7-Diethanolaminomethylene-10, 11-ethylenedioxy-20(R,S)-camptothecin,
18.	7-Diethanolaminomethylene-10, 11-ethylenedioxy-20(S)-camptothecin,
19.	7-Azetidinomethylene-10, 11-methylenedioxy-20(R,S)-camptothecin,
20.	7-Azetidinomethylene-10, 11-methylenedioxy-20(S)-camptothecin,
21.	7-Thiomorpholinomethylene-10, 11-ethylenedioxy-20(S)-camptothecin,
22.	7-Azetidinomethylene-10, 11-ethylenedioxy-20(S)-camptothecin,
23.	7-(4-Methylpiperazinomethylene)-10, 11-methylenedioxy-20(S)-camptothecin,
24.	7-Trifluoroacetamidomethylene-10, 11-ethylenedioxy-20(S)-camptothecin
25.	7-Trifluoroacetamidomethylene-10, 11-methylenedioxy-20(S)-camptothecin
26.	7-Aminomethylene-10, 11-ethylenedioxy-20(S)-camptothecin dihydrochloride,
27.	7-Aminomethylene-10, 11-methylenedioxy-20(S)-camptothecin dihydrochloride,
28.	7-tert-Butyloxycarbonyl-piperazinomethylene-10, 11-ethylenedioxy-20(S)-camptothecin,
29.	7-Piperazinomethylene-10, 11-ethylenedioxy-(S)-camptothecin trifluoroacetic acid salt,
30.	7-(α,α,α -Trifluoro-m-tolyl)-piperazinomethylene-10, 11-ethylenedioxy-20(S)-camptothecin,
31.	7-(2-Methoxyphenyl-piperazino)methylene-10, 11-ethylenedioxy-20(S)-camptothecin and
32.	7-Phenylpiperazinomethylene-10, 11-ethylenedioxy-20(S)-camptothecin

Preparation of Compounds

The compounds of the present invention may be prepared by the procedure shown in Scheme I:

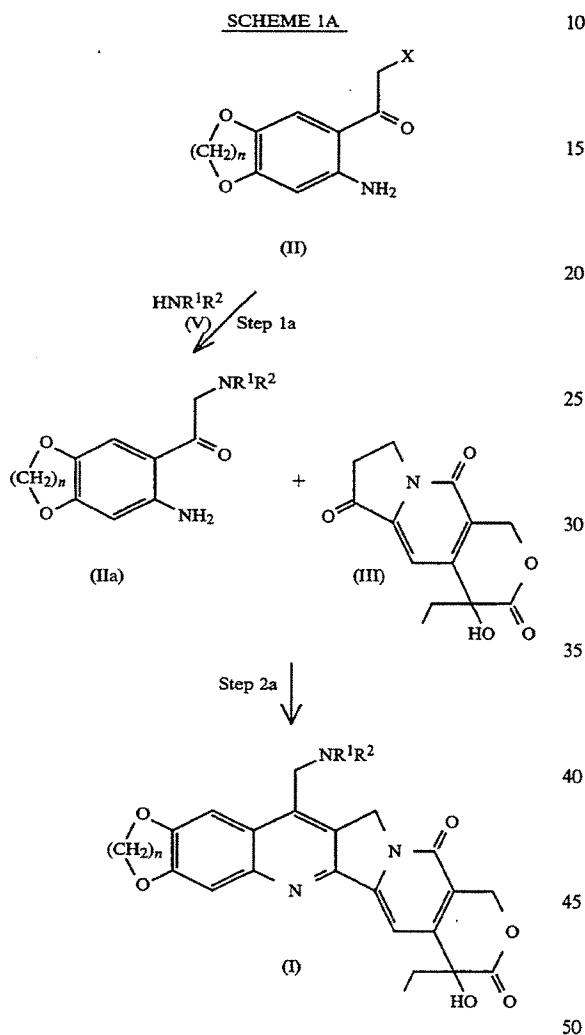


In Step 1 of Scheme I, a compound of formula (II), wherein X is a leaving group (as defined in J. March, *Advanced Organic Chemistry*, 3rd. Ed., page 179, John Wiley & Sons, New York (1985)), for example, a halogen, e.g., chloro, may be reacted with a compound of formula (III) according to the method taught in U.S. Pat. No. 4,894,456 (hereinafter, '456), issued Jan. 16, 1990 to Wall et al., incorporated herein by reference, to yield a compound of formula (IV).

In Step 2, i.e., general process (A), the compounds of formula (IV) may be converted to the compounds of formula (I) by displacement of the leaving group, X, with a compound of formula (V), wherein R¹ and R² are as defined for formula (I). This displacement reaction may conveniently be carried out in a solvent system, for example water, a (C₁₋₄) alkanol, a (C₂₋₄) alkylene diol, 1-hydroxy-2-methoxyethane, dimethylacetamide (DMAC), N-methylpyrrolidinone, dimethyl formamide (DMF), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), toluene or a combination of these solvents in the presence of excess amine, i.e., excess compound of formula (V), with or without a base, e.g., potassium carbonate.

This method is particularly useful for preparing compounds of formula (I) wherein neither R¹ nor R² are hydrogen.

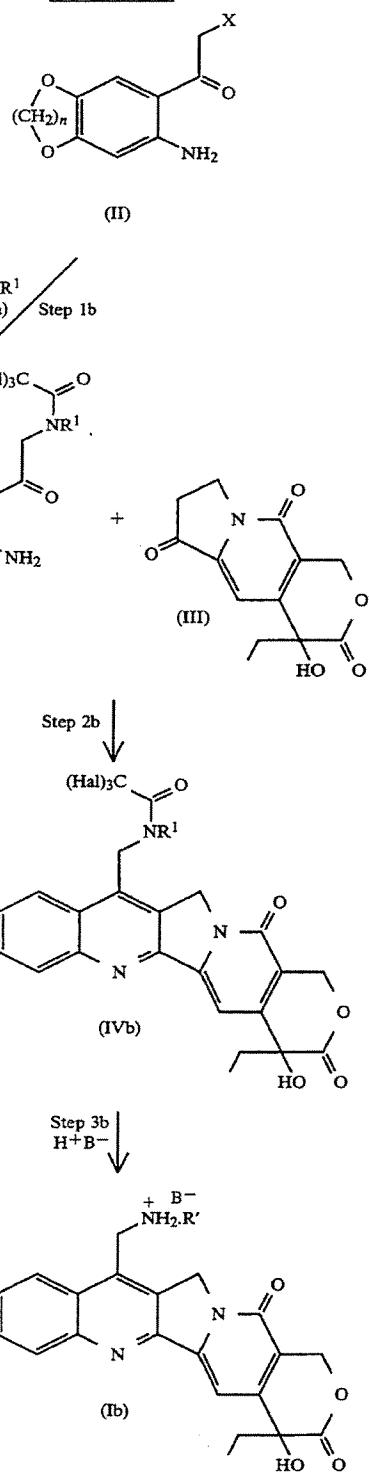
An alternate method for preparing the compounds of the present invention is shown in Scheme 1A:



In Step 1a, a compound of formula (V) is reacted with a compound of formula (II) to yield a compound of formula (IIA), wherein R¹ and R² are as defined for the compounds of formula (I). This reaction may be carried out under conditions similar to those described in Scheme 1, Step 2.

In Step 2a, general process (B), compound of formula (IIA) is reacted with a compound of formula (III) in a similar manner to that taught above in Scheme 1, Step 1, to yield a compound of formula (I).

Another alternate method for preparing the compounds of the present invention is shown in Scheme 1B

SCHEME 1B

In Step 1b, a compound of formula (Va) (wherein "Hal" is halogen, i.e., fluoro, chloro, bromo or iodo) e.g., trifluoroacetamide, is reacted with a compound of formula (II) in a polar, aprotic solvent, e.g., acetonitrile, in the presence of a base soluble in the polar, aprotic

solvent, e.g., cesium carbonate if the solvent is acetonitrile, to yield a compound of formula (IIb).

In Step 2b, a compound of formula (IIb) is reacted with a compound of formula (III) in a similar manner to that taught in Scheme 1, Step 1, yield a compound of formula (IVb).

In Step 3b, general process (C), a compound of formula (IVb) is treated with an acid, $H^+ + B^-$, such as a mineral acid, e.g., hydrochloric acid or sulfuric acid, to yield a compound of formula (Ib), i.e. salt of a compound of formula (I). The compound of formula (Ib) may be treated with a base, such as an alkali metal hydroxide or carbonate, e.g., sodium hydroxide or potassium carbonate, by standard method of the art to yield the corresponding free base. For example a compound of formula (Ib) may be stirred with an aqueous solution of potassium carbonate for about one to about four hours in the temperature range of from about 5° to about 100° C. The free base can then be converted by conventional means to a pharmaceutically acceptable salt if required.

This alternate method is particularly useful for preparing compounds of Formula (I) wherein both R^1 and R^2 are hydrogen or R^2 is hydrogen.

The compounds of formula (II) may be prepared according to the procedure shown in Scheme II:

art, such as those taught in T. Green, *Protective Groups in Organic Chemistry*, Chap. 7, John Wiley, New York (1981). For example, a compound of formula (IX) may be heated at reflux in concentrated hydrochloric acid, and the resulting salt neutralized with a base, e.g., sodium hydroxide, to yield a compound of formula (II).

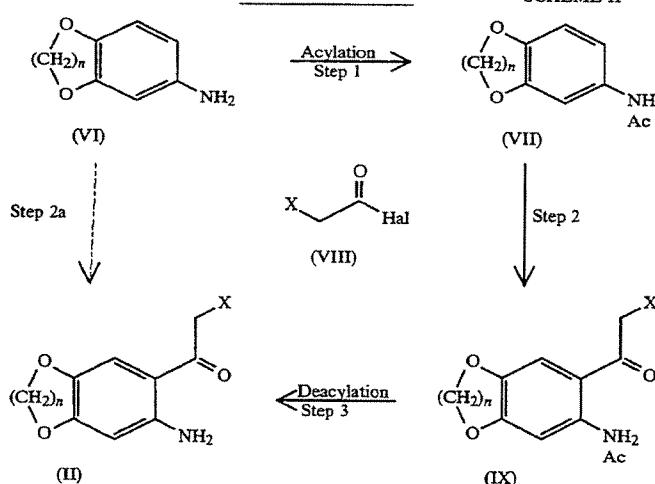
In Step 2a of Scheme II, when the compound of formula (VI) is the ethylenedioxy compound, i.e., n is equal to 2, it may be reacted directly with a compound 10 of formula (VIII), without first protecting the amino group by acylation, to yield the corresponding compound of formula (II).

Alternatively compounds of formula (II) may be prepared according to the method taught by T. Sugasawa, et al., *J. Org. Chem.*, **44**, 578 (1979).

The compound of formula (III) may be prepared according to the procedure of Wall, et al., '456, at column 11, starting at line 30. It is apparent from Scheme 1 that the configuration of the asymmetric carbon of the 20 compound of formula (III) will govern the configuration of the compounds of formula (I). The racemic compound of formula (III) can be resolved into either of its enantiomers by the method of Wani, et al., in U.S. Pat. No. 5,053,512, (hereinafter, "512") incorporated herein by reference.

The novel, intermediate compounds of formulas (II),

SCHEME II



In Step 1 of Scheme II, a compound of formula (VI) is reacted with an acylating agent, for example, a (C_{2-5}) alkanoic acid halide or (C_{2-5}) alkanoic acid anhydride, e.g., acetyl chloride or acetic anhydride, in the presence of a weak base, for example, potassium carbonate, in a polar, aprotic, solvent, for example, chloroform, to yield a compound of formula (VII), wherein Ac is a (C_{2-5}) acyl group.

In Step 2, a compound of formula (VII) is reacted with a compound of formula (VIII), wherein X is a leaving group as defined for the compounds of formula (IV) and Hal is halogen, in the presence of a metallic halide, e.g., zinc chloride, in a polar, aprotic solvent, e.g., nitromethane, to yield a compound of formula (IX). A compound of formula (VIII), for example, may be a haloacetyl halide, e.g., chloroacetyl chloride, or a haloacetonitrile, e.g., chloroacetonitrile.

In Step 3, a compound of formula (II) is formed by removal of the acyl group, Ac, from a compound of formula (IX), i.e., deacylation, by methods known in the

(IIA) and (IV) are within the scope of this invention.

According to another general process (D), a compound of formula (I) according to the invention may be converted into another compound of the invention using conventional procedures.

Thus, for example, a compound of formula (I) wherein one or more of R^1 and R^2 represent a hydrogen atom, may be alkylated using conventional techniques. The reaction may be effected using a suitable alkylating agent such as an alkyl halide, an alkyl tosylate or a dialkylsulphate. the alkylation reaction may conveniently be carried out in an organic solvent such as an amide, e.g. dimethylformamide, or an ether, e.g. tetrahydrofuran, preferably in the presence of a base. Suitable bases include, for example, alkali metal hydrides, such as sodium hydride, alkali metal carbonates, such as sodium carbonate, or potassium methoxide, ethoxide or t-butoxide. The alkylation reaction is conveniently car-

ried out at a temperature of from about 25° to about 100° C.

Alternately, a compound of formula (I) wherein one or more of R¹ and R² represents a hydrogen atom may be converted to another compound of formula (I) by reductive alkylation. Reductive alkylation with an appropriate aldehyde or ketone may be effected using an alkaline earth metal borohydride or cyanoborohydride. The reaction medium, conveniently in an alcohol, e.g. methanol or ethanol or an ether, e.g. dioxan or tetrahydrofuran, optionally in the presence of water. The reaction may conveniently be carried out at a temperature in the range of 0° to 100° C., preferably about 5° to about 50° C.

Alternatively, a compound of formula (I) wherein one or more of R¹ and R² represents a lower alkenyl group may be converted to another compound of formula (I) wherein R¹ and R² represents a lower alkyl group. Reduction may conveniently be effected in the presence of hydrogen and a metal catalyst, for example, Raney nickel or a noble metal catalyst such as palladium, platinum, platinum oxide or rhodium, which may be supported, for example, on charcoal. The reaction may be effected in a solvent such as an alcohol, for example ethanol and conveniently at a temperature of from about -10° to about +50° C., preferably about 20° to about 30° C.

According to another general process (E), a compound of formula (I) according to the invention, or a salt thereof may be prepared by subjecting a protected derivative of formula (I) or a salt thereof to reaction to remove the protecting group or groups.

Thus, at an earlier stage in the preparation of a compound of formula (I) or a salt thereof it may have been necessary and/or desirable to protect one or more sensitive groups in the molecule to prevent undesirable side reactions.

The protecting groups used in the preparation of compounds of formula (I) may be used in conventional manner. See for example, "Protective Groups in Organic Chemistry" Ed. J.F.W. McOmie (Plenum Press 1973) or "Protective Groups in Organic Synthesis" by Theodora W. Greene (John Wiley and Sons 1981).

Conventional amino protecting groups may include, for example, aralkyl groups, such as benzyl, diphenylmethyl or triphenylmethyl groups; and acyl groups such as N-benzyloxycarbonyl or t-butoxycarbonyl. Thus, compounds of general formula (I) wherein one or more of the groups R¹ and R² represent hydrogen may be prepared by deprotection of a corresponding protected compound.

Hydroxy groups may be protected, for example, by aralkyl groups, such as benzyl, diphenylmethyl or triphenylmethyl groups, acyl groups, such as acetyl, silicon protecting groups, such as trimethylsilyl or t-butyl dimethylsilyl groups or as tetrahydropyran derivatives.

Removal of any protecting groups present may be achieved by conventional procedures. Thus, an aralkyl groups such as benzyl, may be cleaved by hydrogenolysis in the presence of a catalyst (e.g. palladium on charcoal); an acyl group such as N-benzyloxycarbonyl may be removed by hydrolysis with, for example, hydrogen bromide in acetic acid or by reduction, for example by catalytic hydrogenation; silicon protecting groups may be removed, for example, by treatment with fluoride ion or by hydrolysis under acidic conditions; tetrahydropyran groups may be cleaved by hydrolysis under acidic conditions.

As will be appreciated, in any of the general processes (A) to (D) described above, it may be necessary or desired to protect any sensitive groups in the molecule as just described. Thus, a reaction step involving deprotection of a protected derivative of general formula (I) or a salt thereof may be carried out subsequent to any of the above described processes (A) to (D).

Thus, according to a further aspect of the invention, the following reactions may, if necessary and/or desired be carried out in any appropriate sequence subsequent to any of the processes (A) to (D)

- (i) removal of any protecting groups; and
- (ii) conversion of a compound of formula (I) or a salt thereof into a pharmaceutically acceptable salt thereof.

Where it is desired to isolate a compound of the invention as a salt, for example, as an acid addition salt, this may be achieved by treating the free base of general formula (I) with any appropriate acid, preferably with an equivalent amount, or with creatinine sulphate in a suitable solvent (e.g. aqueous ethanol).

As well as being employed as the last main step in the preparative sequence, the general methods indicated above for the preparation of the compounds of the invention may also be used of the introduction of the desired groups at an intermediate stage in the preparation of the required compound. It should therefore be appreciated that in such multi-stage processes, the sequence of reactions should be chosen in order that the reacting conditions do not affect groups present in the molecule which are desired in the final product.

The biological activity of the compounds of formula (I) appears to reside in the S enantiomer, and the R enantiomer has little or no activity. Thus, the S enantiomer of a compound of formula (I) is generally preferred over a mixture of R and S such as the racemic mixture. However, if the R enantiomer were desired, e.g., for control studies or synthesis of other compounds, it could be conveniently prepared by the procedure above using the R enantiomer of the compound of formula (III) prepared according to the teachings of '512.

A compound of formula (I) prepared by reaction Scheme I or Scheme II, be purified by conventional methods of the art, e.g., chromatography, distillation or crystallization.

Cleavable Complex in vitro Assay

The data in Table A, below, shows the relative topoisomerase Type I inhibitory activity of the compounds of Formula (I). This assay performed according to the method described in Hsiang, Y. et al., *J. Biol. Chem.*, 260:14873-14878 (1985), correlates well with in vivo anti-tumor activity of topoisomerase inhibitors in animal models of cancer, e.g., camptothecin and its analogs. See Hsiang et al., *Cancer Research*, 49:4385-4389 (1989) and Jaxel et al., *Cancer Research*, 49:1465-1469 (1989).

Those compounds which exhibit observable activity at concentrations greater than 2000 nM ("+" in Table A) are considered weakly to moderately active, while those with activity at concentrations less than 500 nM ("+++" in Table A) are very active. The term "IC₅₀" means the concentration of a compound of formula (I) at which 50% of the DNA substrate has been captured by topoisomerase I.

TABLE A

Topoisomerase Inhibitory Activity of

TABLE A-continued

Example Number	Isomeric form	Relative IC ₅₀ *
2	(S)	++++
6	(S)	++++
11	(S)	++++
1	(R,S)	+++
17	(R,S)	+++
5	(R,S)	+++
4	(S)	++
.9	(R,S)	++
10	(R,S)	++
13	(R,S)	++
16	(R,S)	++
7	(R,S)	++
15	(R,S)	++
16	(R,S)	++
19	(R,S)	++
8	(R,S)	+

***IC₅₀ Range**

Symbol	nM
++++	<~500
+++	<~1000 >~500
++	<~2000 >~1000
+	>~2000

The compounds of formula (IV) have also been found to have good topoisomerase I inhibitory activity.

Human Tumor Xenografts

In recent years, human tumor xenografts heterotransplanted into nude mice have been widely used to assess the antitumor activities of cancer chemotherapeutic agents. See Giovannella, B. C., Stehlin, Jr., J. S., Shepard, R. C. and Williams, Jr., L. J., "Correlation between response to chemotherapy of human tumors in patients and in nude mice", *Cancer* 52:1146-1152, (1983); Boven, E. and Winograd, B., Eds. *The Nude Mouse in Oncology Research* CRC Press, Inc., Boca Raton, FL, (1991); and Fiebig, H. H., "Comparison of tumor response in nude mice and in patients", *Human Tumour Xenografts in Anticancer Drug Development*, Winograd, B., Peckham, M. J., and Pinedo, H. M., Eds., E.S.O. Monographs, Springer, Heidelberg, 25 (1988).

In general, human tumor xenografts retain not only the histological, biochemical and antigenic characteristics, but also the chemosensitivity of the tumor tissue of origin (Boven, et al., supra). Lengthy studies have provided evidence that human tumor xenografts retain these important biological properties of the tumor of origin including a biological instability as is known to occur in patient's tumors (Boven, et al., supra). Most importantly, several investigators have reported good correlations between drug effects in the human tumor xenografts and clinical results in human patients (Giovannella, et al., and Fiebig supra).

Human Colorectal Adenocarcinoma HT-29 Xenograft in vivo Assay

Female NU/NU mice weighing 21±2 g, are used for this modified version of the test described by B. C. Giovannella, et al., *Science*, 246 1046 (1989). Control and test animals are injected subcutaneously in the subscapular region with a suspension of 10⁶ viable HT-29 human colon tumor cells on day 0. Tumors are allowed to grow for 2 weeks prior to drug administration. For each drug, several doses are chosen based on its in vitro activity against topoisomerase I. Each dose level group contains 8 animals. The test compounds are prepared in either 0.1M acetate buffer, pH 5 (vehicle "a") or 87.5%

phosphate buffered saline, 12.375% dimethylsulfoxide, and 0.125% Tween 80 (trademark of ICI America for polyoxyethylenesorbitan monooleate) (vehicle "b") and are administered subcutaneously twice a week for 5 weeks beginning on day 14. Doses are given on a mg/kg basis according to the mean body weight for each cage.

Tumor weight is calculated from two perpendicular caliper measurements of the tumor using the formula, tumor weight= length × width² + 2 in millimeters. For each animal, tumor weight is monitored over the course of the experiment. For each group, the results are expressed as the ratio of the mean tumor weight immediately after 5 weeks of treatment (day 50) divided by the mean tumor weight immediately before treatment (day 14). Results are expressed in Table B. For either of the vehicle controls, the ratio is approximately 20, indicating that the tumor, in the absence of drug treatment, increased in weight approximately 20-fold over the course of the experiment. In contrast, a ratio of 1 indicates tumor stasis while a ratio less than 1 indicates tumor regression. Thus, compounds 4 and 6 caused tumor stasis while compounds 11 and 23 caused tumor regression. The criterion for antitumor activity is at least 50% inhibition of tumor growth after 5 weeks of dosing (day 50), giving a ratio of less than or equal to ten.

TABLE B

Compound	Optimal Dose in vivo Antitumor activity	
	(mg/kg)	(tumor wt _{day 50} /tumor wt _{day 14})
control (vehicle alone)	—	20.0 ^a , 21.8 ^b
2	0.8	1.8 ^b
4	7.0	1.3 ^a , 1.0 ^b
6	1.0	1.0 ^b
11	9.0	0.6 ^a
14	2.0	2.0 ^a
20	1.5	1.5 ^a
22	12.0	1.6 ^a
23	3.0	0.5 ^a

^avehicle of 0.1M acetate buffer, pH 5.

^bvehicle of 87.5% phosphate buffered saline, 12.375% dimethylsulfoxide, and 0.125% Tween 80.

Utility

In view of such activity, the compounds of formula (I) are active against a wide spectrum of mammalian (including human) tumors and cancerous growths such as cancers of the oral cavity and pharynx (lip, tongue, mouth, pharynx), esophagus, stomach, small intestine, large intestine, rectum, liver and biliary passages, pancreas, larynx, lung, bone, connective tissue, skin, colon, breast, cervix uteri, corpus endometrium, ovary, prostate, testis, bladder, kidney and other urinary tissues, eye, brain and central nervous system, thyroid and other endocrine gland, leukemias (lymphocytic, granulocytic, monocytic), Hodgkin's disease, non-Hodgkin's lymphomas, multiple myeloma, etc. Herein the terms "tumor", "cancer" and "cancerous growths" are used synonymously.

The amount of compound of formula (I) required to be effective as an antitumor agent will, of course, vary with the individual mammal being treated and is ultimately at the discretion of the medical or veterinary practitioner. The factors to be considered include the condition being treated, the route of administration, the nature of the formulation, the mammal's body weight, surface area, age and general condition, and the particular compound to be administered. However, a suitable

effective antitumor dose is in the range of about 0.1 to about 200 mg/kg body weight per day, preferably in the range of about 1 to about 100 mg/kg per day. The total daily dose may be given as a single dose, multiple doses, e.g., two to six times per day, or by intravenous infusion for a selected duration. Dosages above or below the range cited above are within the scope of the present invention and may be administered to the individual patient if desired and necessary.

For example, for a 75 kg mammal, a dose range 10 would be about 75 to about 7500 mg per day, and a typical dose would be about 800 mg per day. If discrete multiple doses are indicated, treatment might typically be 200 mg of a compound of formula (I) given 4 times per day.

Formulations

Formulations of the present invention, for medical use, comprise an active compound, i.e., a compound of formula (I), together with an acceptable carrier thereof and optionally other therapeutically active ingredients. The carrier must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The present invention, therefore, further provides a pharmaceutical formulation comprising a compound of formula (I) together with a pharmaceutically acceptable carrier thereof.

The formulations include those suitable for oral, rectal or parenteral (including subcutaneous, intramuscular and intravenous) administration. Preferred are those suitable for oral or parenteral administration.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the active compound into association with a carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing 35 the active compound into association with a liquid carrier or a finely divided solid carrier and then, if necessary, shaping the product into desired unit dosage form.

Formulations of the present invention suitable for oral administration may be presented as discrete units 45 such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the active compound; as a powder or granules; or a suspension or solution in an aqueous liquid or non-aqueous liquid, e.g., a syrup, an elixir, an emulsion or a draught.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active compound in a free-flowing form, e.g., a powder or granules, optionally mixed 55 with accessory ingredients, e.g., binders, lubricants, inert diluents, surface active or dispersing agents. Molded tablets may be made by molding in a suitable machine, a mixture of the powdered active compound with any suitable carrier.

A syrup or suspension may be made by adding the active compound to a concentrated, aqueous solution of a sugar, e.g., sucrose, to which may also be added any accessory ingredients. Such accessory ingredient(s) may include flavoring, an agent to retard crystallization 65 of the sugar or an agent to increase the solubility of any other ingredient, e.g., as a polyhydric alcohol, for example, glycerol or sorbitol.

Formulations for rectal or vaginal administration may be presented as a suppository with a conventional carrier, e.g., cocoa butter or Witepsol S55 (trademark of Dynamite Nobel Chemical, Germany, for a suppository base).

For transdermal administration, the compounds according to the invention may be formulated as creams, gels, ointments or lotions or as a transdermal patch. Such compositions may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening, gelling, emulsifying, stabilizing, dispersing, suspending and/or coloring agents.

Formulations suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the active compound which is preferably isotonic with the blood of the recipient. Such formulations suitably comprise a solution or suspension of a pharmaceutically and pharmacologically acceptable acid addition salt of a compound of the formula (I) that is isotonic with the blood of the recipient. Thus, such formulations may conveniently contain distilled water, 5% dextrose in distilled water or saline and a pharmaceutically and pharmacologically acceptable acid addition salt of a compound of the formula (I) that has an appropriate solubility in these solvents, for example the hydrochloride. Useful formulations also comprise concentrated solutions or solids containing the compound of formula (I) which upon dilution with an appropriate solvent give a solution suitable for parenteral administration above.

In addition to the aforementioned ingredients, the formulations of this invention may further include one or more optional accessory ingredient(s) utilized in the art of pharmaceutical formulations, e.g., diluents, buffers, flavoring agents, binders, surface active agents, thickeners, lubricants, suspending agents, preservatives (including antioxidants) and the like.

EXAMPLES

The following examples illustrate aspects of this invention but should not be construed as limitations. The symbols and conventions used in these examples are consistent with those used in the contemporary chemical literature, for example, the *Journal of the American Chemical Society*. As used here in the term "room temperature" means about 25° C.

EXAMPLE 1

7-Dimethylaminomethylene-10,
11-methylenedioxy-20(R,S)-camptothecin (Compound
1)

(A) 3,4-Methylenedioxyacetanilide

To commercially available 3,4-methylenedioxy aniline (17.0 g, 124 mmol) and sodium carbonate (15.5 g, 136 mmol) in chloroform (90 mL) at 5° C. is added acetyl chloride (8.8 g, 124 mmol) dropwise with stirring. The reaction is allowed to warm to room temperature and stirring is continued for about 18 hours. The reaction mixture is washed twice with about 50 mL of 1N HCl and the organic layer is dried ($MgSO_4$) and the solvent removed to yield a brown solid. Recrystallization from water with activated carbon treatment yields 3,4-methylenedioxyacetanilide (9.34 g, 42.1% of theory) as a light brown solid. Elemental analysis: ($C_9H_9NO_3$):

	% C	% H	% N
Found:	60.34	5.04	7.79
Calculated	60.33	5.06	7.82

(B)

2'-Acetylamino-4',5'-methylenedioxy-2-chloroacetophenone

To a mixture of zinc chloride (24.3 g, 178.3 mmol) and chloroacetylchloride (16.1 mL, 202.1 mmol) in nitromethane (85 mL), under nitrogen, at room temperature, with stirring, is added, dropwise, 3,4-methylenedioxycetanilide (8.96 g, 50.0 mmol) in nitromethane (15 mL). This mixture is then heated at reflux for 1.5 hrs, allowed to cool to room temperature, poured over ice, extracted with methylene chloride, which is then removed by evaporation, to yield a brown solid. This solid is recrystallized from an ethyl acetate/hexane mixture (including treatment with activated charcoal) to yield 2'-acetylamino-4',5'-methylenedioxy-2-chloroacetophenone (831.3 mg, 6.5% of theory) as yellow crystals. ¹H-NMR (CDCl₃): δ 8.45 (s, 1H); 7.2 (s, 1H); 6.09 (s, 2H); 4.65 (s, 2H); 2.25 (s, 3H).

(C) 3,4-Methylenedioxypivaloylanilide

This compound is prepared by the method of Example 1(a) except an equivalent amount of 2,2-dimethylpropanoyl chloride is used in place of acetyl chloride.

(D)

2'-Pivoylamino-4',5'-methylenedioxy-2-chloroacetophenone

This compound is prepared by the method of Example 1(B) except an equivalent amount of 3,4-methylenedioxypivaloylanilide is used in place of 3,4-methylenedioxycetanilide.

(E)

2'-Amino-4',5'-methylenedioxy-2-chloroacetophenone

To 2'-acetylamino-4',5'-methylenedioxy-2-chloroacetophenone (0.9 g, 3.53 mmol) or an equivalent amount of 2'-pivoylamino-4',5'-methylenedioxy-2-chloroacetophenone in ethanol (60 mL) at about 5° C. is added, dropwise, conc. HCl (12.5 mL, 149.7 mmol). The reaction mixture is then heated at reflux for about an hour, then poured over 2N NaOH/ice (80 mL/60 g) and washed with ethyl acetate (3×70 mL). The organic portions are combined and washed with brine (50 mL), dried (anhydrous sodium sulfate) and concentrated in vacuo to yield a greenish-yellow solid. This solid is recrystallized from ethyl acetate/isopropanol/hexane, treated with activated charcoal, to yield 2'-amino-4',5'-methylenedioxy-2-chloroacetophenone (0.39 g, 52% of theory).

Elemental analysis: (C₉H₈NO₃Cl)

	% C	% H	% N
Found:	50.66	3.80	6.47
Calculated	50.60	3.77	6.56

(F)

5'(R,S)-1,5-Dioxo-(5'-ethyl-5'-hydroxy-2'H,5'H,6'H-oxopyrano)[3', 4'-f]Δ^{6,8}-tetrahydroindolizine and 5'(S)-1,5-Dioxo-(5'-ethyl-5'-hydroxy-2'H,5'H,6'H-6-oxopyrano)[3', 4'-f]Δ^{6,8}-tetrahydroindolizine (compounds of formula (III))

These compounds, referred to hereinafter as "tricyclic ketone (R,S)" and "tricyclic ketone (S)" respectively or collectively as "a compound of formula (III)", are prepared according to the procedure taught by Wani et al., in '512. Note that the corresponding R enantiomer may also be prepared by the procedure of '512.

(G)

7-Chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin

Following the general procedure for camptothecin taught in '512, 2'-amino-4',5'-methylenedioxy-2-chloroacetophenone is stirred in refluxing toluene (50 mL) with tricyclic ketone (R,S) (256.3 mg, 0.97 mmol) under a Dean-Stark trap for half an hour. The reaction is then cooled and the solid filtered and washed with toluene and ethanol to yield 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin, (408.5 mg, 68.8%). ¹H-300 NMR (DMSO-d₆): δ 7.72 (s, 1H); 7.55 (s, 1H); 7.2 (s, 1H); 6.34 (s, 2H); 5.42 (s, 2H); 5.32 (s, 2H); 5.24 (s, 2H); 1.85 (m, 2H); 0.88 (t, 3H). Nominal Mass Spectrum M+1: Calcd.: 441 Found: 441

(H) 7-Dimethylaminomethylene-10,11-methylenedioxy-20(R,S)-camptothecin

To a stirred mixture of 7-chloromethyl-10, 11-methylenedioxy-(R,S)-camptothecin (0.11 g, 0.25 mmol) and potassium carbonate (346 mg, 0.5 mmol) in dimethylformamide (DMF) (1 mL) is added dimethylamine (6.1 mL, 0.5 mmol) in the form of a 3.73 mg/mL solution in tetrahydrofuran at about 5° C. The reaction mixture is securely stoppered, allowed to warm to room temperature, stirred for about 15 hrs and then filtered to remove the solid material. The filtrate is concentrated by vacuum evaporation and the resulting solid triturated with acetonitrile and filtered. The filtrate is concentrated by vacuum evaporation to a thick residue. The residue is dissolved in minimal amount of chloroform and chromatographed on 30 grams of flash grade silica gel eluting with successive portions of 250 mls of ethyl acetate followed by 250 mls of (9:1 ethyl acetate, isopropanol) finally with 250 mls of (4:1 ethyl acetate, isopropanol). Fractions were collected and monitored by TLC (5% methanol, ethyl acetate) and visualized by a UV lamp. The appropriate fractions were pooled, concentrated and dried under vacuo to yield 7-dimethylaminomethylene-10, 11-methylenedioxy-20(R,S)-camptothecin (6.0 mg, 4.7%). This compound was characterized as its acetic acid salt.

m.p. >250° C. Elemental analysis: (C₂₄H₂₃N₃O₃C₂H₄O₂):

	% C	% H	% N
Found:	61.64	5.17	8.73
Calculated	61.29	5.34	8.25

(I) Open E ring form

The compound of part (H) is treated with an equivalent amount of sodium hydroxide to form the corresponding open E ring form. Treatment of the latter with an equivalent amount of hydrochloric acid closes the E ring and thereby reforms the compound of part (H). 5

EXAMPLE 2

7-Dimethylaminomethylene-10,
11-methylenedioxy-20(S)-camptothecin (Compound 2)

(A)

7-Chloromethyl-10,11-methylenedioxy-20(S)-camptothecin

This compound is prepared by the procedure of Example 1, except in part (G) an equivalent amount of tricyclic ketone (S) is used in place of tricyclic ketone (R,S).

m.p. > 250° C.

(B) 7-Dimethylaminomethylene-10,
11-methylenedioxy-20(S)-camptothecin

This compound is prepared by the procedure of Example 1, part (H), except that an equivalent amount of 7-chloromethyl-10, 11-methylenedioxy-20(S)-camptothecin, prepared according Example 2, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

m.p. > 250° C.

Nominal Mass Spectrum M+1: Calcd.: 450. Found: 450.

EXAMPLE 3

7-Dimethylaminomethylene-10,
11-ethylenedioxy-20(R,S)-camptothecin (Compound 3)

(A)

7-Chloromethyl-10,11-ethylenedioxy-20(R,S)-camptothecin

This compound is prepared by the procedure of Example 1, except in parts (A) and (C) an equivalent amount of 3,4-ethylenedioxy aniline is used in place of 3,4-methylenedioxy aniline.

High Resolution Mass Spectrum M+1: Calcd.: 455.1009. Found: 455.1005

(B) 7-Dimethylaminomethylene-10,
11-ethylenedioxy-20(R,S)-camptothecin

This compound is prepared by the procedure of Example 1, part (H), except that an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(R,S)-camptothecin is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum: Calcd.: 464.1821. Found: 464.1833.

EXAMPLE 4

7-Dimethylaminomethylene-10,
11-ethylenedioxy-20(S)-camptothecin (Compound 4)

(A)

7-Chloromethyl-10,11-ethylenedioxy-20(S)-camptothecin

This compound is prepared by the procedure of Example 1 except in parts (A) and (C) an equivalent amount of 3,4-ethylenedioxy aniline is used in place of 3,4-methylenedioxy aniline, and in part (G) an equiva-

lent amount of tricyclic ketone (S) is used in place of tricyclic ketone (R,S)

High Resolution Mass Spectrum M+1: Calcd.: 455.1009. Found: 455.1000.

(B) 7-Dimethylaminomethylene-10,
11-ethylenedioxy-20(S)-camptothecin

This compound is prepared by the procedure of Example 1, part (H), except that an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(S)-camptothecin in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum: Calcd.: 464.1821. Found: 464.1811.

EXAMPLE 5

7-Morpholinomethylene-10,11-ethylenedioxy-20(R,S)-camptothecin (Compound 5)

20 The same procedure as Example 1, part (H), is used except that an equivalent amount of morpholine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(R,S)-camptothecin, prepared according to Example 3, part (B), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum: Calcd: 506.1942. Found: 506.1925.

EXAMPLE 6

7-Morpholinomethylene-10,11-ethylenedioxy-20(S)-camptothecin (Compound 6)

35 The same procedure as Example 1, part (H), is used except that an equivalent amount of morpholine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(S)-camptothecin, prepared according to Example 4, part (B), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

40 High Resolution Mass Spectrum: Calcd.: 506.1942. Found: 506.1929.

EXAMPLE 7

7-Pyrrolidinomethylene-10,11-ethylenedioxy-20(R,S)-camptothecin (Compound 7)

The same procedure as Example 1, part (H), is used except that an equivalent amount of pyrrolidine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(R,S)-camptothecin, prepared according to Example 3, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum: Calcd.: 490.1978. Found: 490.1988.

EXAMPLE 8

7-Piperidinomethylene-10,11-methylenedioxy-20(R,S)-camptothecin (Compound 8)

The same procedure as Example 1, part (H), is used except that an equivalent amount of piperidine is used in place of dimethylamine.

¹H-300 NMR (DMSO-d₆): δ 7.95 (s, 1H); 7.62 (s, 1H); 7.29 (s, 1H); 6.35 (s, 2H); 5.49 (s, 2H); 5.41 (s, 2H); 4.85 (broad s, 2H); 1.9–0.7 (m, 11H).

Nominal Mass Spectrum M+1: Calcd.:490. Found: 490.

21**EXAMPLE 9**

7-Piperidinomethylene-10,
11-ethylenedioxy-20(R,S)-camptothecin (Compound 9)

The same procedure as Example 1, part (H), is used except that an equivalent amount of piperidine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(R,S)-camptothecin, prepared according to Example 3, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum:
Calcd.: 504.2127. Found: 504.2129.

EXAMPLE 10

7-(4-methylpiperazinomethylene)-10,
11-ethylenedioxy-20(R,S)-camptothecin (Compound 10)

The same procedure as Example 1, part (H), is used except that an equivalent amount of 4-methylpiperazine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(R,S)-camptothecin, prepared according to Example 3, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum: Calcd.: 519.2236.
Found: 519.2246.

EXAMPLE 11

7-(4-methylpiperazinomethylene)-10,
11-ethylenedioxy-20(S)-camptothecin (Compound 11)

The same procedure as Example 1, part (H), is used except that an equivalent amount of 4-methylpiperazine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(S)-camptothecin, prepared according to Example 4, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

m.p. 261°-264° C.

Nominal Mass Spectrum M+1: Calcd.: 519. Found: 519.

EXAMPLE 12

7-Diethylaminomethylene-10,
11-ethylenedioxy-20(S)-camptothecin (Compound 12)

This compound is prepared by the procedure of Example 1, part (H), except that equivalent amount of diethylamine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-methylenedioxy-20(S)-camptothecin, prepared according to Example 2, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum: Calcd.: 478.1978.
Found: 478.1963.

EXAMPLE 13

7-Diethylaminomethylene-10,
11-ethylenedioxy-20(R,S)-camptothecin (Compound 13)

The same procedure as Example 1, part (H), is used except that an equivalent amount of diethylamine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(R,S)-camptothecin, prepared according to Example

22

3, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum
Calcd.: 492.2134
Found: 492.2140

EXAMPLE 14

7-Diethylaminomethylene-10,
11-ethylenedioxy-20(S)-camptothecin (Compound 14)

The same procedure as Example 1, part (H), is used except that an equivalent amount of diethylamine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(S)-camptothecin, prepared according to Example 4, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum: Calcd.: 492.2134.
Found: 492.2122.

EXAMPLE 15

7-N-Methylethanolaminomethylene-10,
11-ethylenedioxy-20(R,S)-camptothecin (Compound 15)

The same procedure as Example 1, part (H), is used except that an equivalent amount of N-methylethanamine is used in place of dimethylamine.

High Resolution Mass Spectrum: Calcd.: 480.1771.
Found: 480.1776.

EXAMPLE 16

7-N-Methylethanolaminomethylene-10,
11-ethylenedioxy-20(R,S)-camptothecin (Compound 16)

The same procedure as Example 1, part (H), is used except that an equivalent amount of N-methylethanamine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(R,S)-camptothecin, prepared according to Example 3, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum: Calcd.: 494.1927.
Found: 494.1929.

EXAMPLE 17

7-Diethanolaminomethylene-10,
11-ethylenedioxy-20(R,S)-camptothecin (Compound 17)

The same procedure as Example 1, part (H), is used except that an equivalent amount of diethanolamine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(R,S)-camptothecin, prepared according to Example 3, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

High Resolution Mass Spectrum: Calcd.: 524.2024.
Found: 524.2026.

EXAMPLE 18

7-Diethanolaminomethylene-10,
11-ethylenedioxy-20(S)-camptothecin (Compound 19)

The same procedure as Example 1, part (H), is used except that an equivalent amount of diethanolamine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(S)-camptothecin, prepared according to Example 4, part

23

(A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

m.p. 230°-233° C.

Nominal Mass Spectrum M + 1: Calcd.: 524. Found: 524.

EXAMPLE 19

7-Azetidinomethylene-10,
11-methylenedioxy-20(R,S)-camptothecin (Compound
19)

The same procedure as Example 1, part (H), is used except that an equivalent amount of azetidine is used in place of dimethylamine.

m.p. >250° C.

Nominal Mass Spectrum M + 1: Calcd.: 462. Found: 462.

EXAMPLE 20

7-Azetidinomethylene-10,
11-methylenedioxy-20(S)-camptothecin (Compound
20)

This compound is prepared by the procedure of Example 1 except in parts (G) an equivalent amount of tricyclic ketone (S) is used in place of tricyclic ketone (R,S), and in part (H) an equivalent amount of azetidine is used in place of dimethylamine.

High Resolution Mass Spectrum: Calcd.: 462.1665.
Found: 462.1667.

EXAMPLE 21

7-Thiomorpholinomethylene-10,
11-ethylenedioxy-20(S)-camptothecin (Compound 21)

The same procedure as Example 1, part (H), is used except that an equivalent amount of thiomorpholine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-methylenedioxy-20(S)-camptothecin, prepared according to Example 4, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

m.p. 249°-252° C.

Nominal Mass Spectrum M + 1: Calcd.: 522. Found: 522.

EXAMPLE 22

7-Azetidinomethylene-10,
11-ethylenedioxy-20(S)-camptothecin (Compound 22)

The same procedure as Example 1, part (H), is used except that an equivalent amount of azetidine is used in place of dimethylamine and an equivalent amount of 7-chloromethyl-10,11-ethylenedioxy-20(S)-camptothecin, prepared according to Example 4, part (A), is used in place of 7-chloromethyl-10,11-methylenedioxy-20(R,S)-camptothecin.

m.p. 208-210 (decomp.)

Low Resolution Mass Spectrum: 476.2 (ES).

EXAMPLE 23

7-(4-Methylpiperazinomethylene)-10,
11-methylenedioxy-20(S)-camptothecin (Compound
23)

This compound is prepared by the procedure of Example 1 except in parts (G) an equivalent amount of 65 tricyclic ketone (S) is used in place of tricyclic ketone (R,S), and in part (H) an equivalent amount of 4-methylpiperazine is used in place of dimethylamine.

24

High Resolution Mass Spectrum: Calcd.: 505.2083.
Found: 505.2087.

EXAMPLE 24

5 7-Trifluoroacetamidomethylene-10,11-ethylenedioxy-
20(S)-camptothecin (Compound 24)

(A)

2'-Amino-4',5'-methylenedioxy-2-tri-
fluoroacetamidoacetophenone

Trifluoroacetamine (227 mg, 2 mmole) is added to a solution of cesium carbonate (1.63 g, 5 mmole) in anhydrous acetonitrile (15 ml) at room temperature under nitrogen.

2'-Amino-4',5'-methylenedioxy-2-chloroacetophenone is then added and the mixture is placed in a preheated oil bath set at 90° C. for 30 minutes. The reaction is cooled to room temperature and poured directly onto a silica plug (15 g) in a scintered glass funnel. The silica is washed two times with EtOAc and the volatiles from the combined washes are removed in vacuo. Diethyl ether is used to triturate the residue to afford a light orange solid which is collected by filtration and dried under vacuum. (498 mg, 86%). Mp=219°-220° C. ¹H NMR (300 MHz, DMSO-d₆): δ 4.44 (d, 2H); 5.96 (s, 2H); 5.96 (s, 2H); 6.35 (s, 1H); 7.21 (s, 1H); 7.40 (bs, 2H); 9.59 (t, 1H). Nominal mass expected: MH⁺ = 291 m/z. found MH⁺ = 291 m/z.

(B)

2'-Amino-4',5'-ethylenedioxy-2-tri-
fluoroacetamidoacetophenone

This compound is prepared as in the method above except an equivalent amount of 2'-amino-4',5'-ethylenedioxy-2-chloroacetophenone is used in place of 2'-amino-4',5'-methylenedioxy-2-chloroacetophenone. A green solid is isolated in 74% yield. Mp=154°-155° C. ¹H NMR (300 MHz, CDCl₃): δ 4.08 (m, 2H); 4.13 (m, 2H); 4.60 (d, 2H); 6.0 (bs, 2H); 6.08 (s, 1H); 7.04 (s, 1H); 7.60 (t, 1H). Nominal mass expected: MH⁺ = 305 m/z. Found: MH⁺ = 305 m/z.

(C)

10,11-Ethylenedioxy-7-trifluoroacetamidomethylene-
20(S)-camptothecin

45 2'-Amino-4',5'-ethylenedioxy-2-tri-
fluoroacetamidoacetophenone (71 mg, 0.234 mmole), tricyclic ketone (S) (61 mg, 0.234 mmole), and anhydrous toluene (2.0 ml) are combined at 60° C. under nitrogen. A catalytic amount of both glacial acetic acid and p-toluenesulfonic acid monohydrate are added before increasing the reaction temperature to reflux. The reaction refluxes for 16 hrs and is then cooled to ambient temperature. A green-yellow solid is collected by filtration, washed with ethanol and diethyl ether, and dried in vacuo. (101 mg, 84%) Mp=249° C. ¹H NMR (300 MHz, DMSO-d₆): δ 0.91 (t, 3H); 1.91 (m, 2H); 4.40 (s, 4H); 4.83 (d, 2H); 5.39 (s, 2H); 5.41 (s, 2H); 6.48 (s, 1H); 7.22 (s, 1H); 7.58 (s, 1H); 7.77 (s, 1H); 10.20 (t, 1H). Nominal mass expected: MH⁺ = 532 m/z. Found: MH⁺ = 532 m/z.

EXAMPLE 25

7-Trifluoroacetamidomethylene-10,11-methylenedioxy-
20(S)-camptothecin (Compound 25)

This compound is prepared by the method of Example 24 above except an equivalent amount of 2'-amino-4',5'-methylenedioxy-2-trifluoroacetamidoacetophenone.

none is used in place of 2'-amino-4',5'-ethylenedioxy-2-trifluoroacetamidoacetophenone. A green-yellow solid is isolated in 15% yield mp=238° C. (d). ¹H NMR (DMSO-d₆): δ 0.91 (t, 3H); 1.95 (m, 2H); 4.92 (s, 2H); 5.38 (s, 2H); 5.40 (s, 2H); 6.28 (s, 2H); 6.49 (s, 1H); 7.13 (s, 1H); 7.58 (s, 1H); 7.78 (s, 1H); 10.21 (t, 1H). Nominal mass expected: MH⁺=518 m/z. Found MH⁺=518 m/z.

EXAMPLE 26

7-Aminomethylene-10,11-ethylenedioxy-20(S)-camptothecin dihydrochloride (Compound 26)

7-trifluoroacetamidomethylene-10,11-ethylenedioxy-20(S)-camptothecin (65 mg, 0.12 mmole) is heated to 105° C. in aqueous 2N hydrochloric acid (1.2 ml) for 20 minutes in an open flask. the volatiles are removed in vacuo and the residue is triturated with ethyl acetate and collected by filtration. The bright yellow solid is washed with ethyl acetate (3 ml), ethanol (2 ml) and diethyl ether (2 ml) and dried in vacuo to afford 62 mg (100%). mp>300° C. ¹H NMR (300 MHz, DMSO-d₆): δ 0.90 (t, 3H); 1.95 (m, 2H); 4.41 (s, 4H); 4.61 (d, 2H); 5.40 (s, 2H); 5.45 (s, 2H); 7.24 (s, 1H); 7.60 (s, 1H); 7.81 (s, 1H); 8.40 (bs, 2H). Nominal mass expected: MH⁺=436 m/z. Found MH⁺=436 m/z.

EXAMPLE 27

7-Aminomethylene-10,11-methylenedioxy-20(S)-camptothecin dihydrochloride (Compound 27)

This compound is prepared by the method of Example 26 above except an equivalent amount of 7-trifluoroacetamidomethylene-10,11-methylenedioxy-20(S)-camptothecin is used in place of 7-trifluoroacetamidomethylene-10,11-ethylenedioxy-20(S)-camptothecin. A golden yellow solid is isolated in quantitative yield. Mp=270° C. (d). ¹H NMR (300 MHz, DMSO-d₆): δ 0.90. (t, 3H); 1.9 (m, 2H); 4.6 (m, 2H); 5.4 (s, 2H); 5.5 (s, 2H); 6.3 (s, 2H); 7.2 (s, 1H); 7.6 (s, 1H); 7.9 (s, 1H); 8.4 (bs, 2H). Nominal mass expected: MH⁺=422 m/z. Found: MH⁺=422 m/z.

EXAMPLE 28

7-tert-Butyloxycarbonyl-piperazinomethylene-10,11-ethylenedioxy-20(S)-camptothecin (Compound 28)

To a -50° C. solution of (S)(-) -10, 11-ethylenedioxy-7-chloromethylcamptothecin (35.8 mg, 78.7×10⁻³ mmol) was added dropwise tert-butyl-l-piperazinecarboxylate (34.6 mg, 186×10⁻³ mmol) in N,N-dimethylformamide (DMF) (0.45 mL). The dark brown reaction mixture was stirred at -50° C. for 10 min, and allowed to warm to 0° C. Additional tert-butyl-l-piperazinecarboxylate (8 mg, 43×10⁻³ mmol) in DMF (0.2 mL) was added, and the mixture was allowed to warm to ambient temperature. The mixture was stirred for an additional 90 min, and the solvent was removed with a rotary evaporator to afford the crude product as a brown residue. Purification by silica gel chromatography (eluting with 100% ethyl acetate) afforded 20.7 mg (58% yield) of the product as a pale yellow solid. ¹H NMR (300 MHz, CDCl₃): δ 1.04 (t, 3H, J=7); 1.45 (s, 9H); 1.87 (m, 2H); 2.46 (s, 4H); 3.41 (s, 4H); 3.94 (s, 2H); 4.43 (s, 4H); 5.29 (s, 2H); 5.30 (d, 1H, J=16); 5.75 (d, 1H, J=16); 7.59 (s, 1H); 7.65 (s, 1H); 7.73 (s, 1H). Nominal mass spectrum (M+1): 605.

EXAMPLE 29

7-Piperazinomethylene-10,11-ethylenedioxy-20(S)-camptothecin trifluoroacetic acid salt (Compound 29)

To a 0° C. solution of 7-tert-butyloxycarbonyl-piperazinomethyl-10, 11-ethylenedioxy-20(S)-camptothecin (16.7 mg, 27.6×10⁻³ mmol) in dry CH₂Cl₂ (5.0 mL) was added trifluoroacetic acid (0.5 mL). The deep yellow solution was allowed to warm to ambient temperature and stirred for 14 h. The mixture was concentrated with a rotary evaporator, and the residue was purified by reverse phase HPLC (Rainin Dynamax 60A column, eluting with 49:10:2.5:1 water/acetonitrile/THF/trifluoroacetic acid) to afford, after concentration and lyophilization of the major UV active peak (monitoring at 254 nm), 21.7 mg of the product as a yellow fluffy powder. ¹H NMR (300 MHz, DMSO-d₆): δ 0.88 (t, 3H, J=7); 1.87 (m, 2H); 2.60–2.80 (m, 4H); 3.00–3.20 (bs, 4H); 5.29 (s, 2H); 5.41 (s, 2H); 6.5 (bs, 1H); 7.25 (s, 1H); 7.56 (s, 1H); 7.80 (s, 1H); 8.50 (bs, 2H). Nominal mass spectrum (M+1): 505. mp: 315° C. (d)

EXAMPLE 30

7-(*a, a, a*-Trifluoro-m-tolyl)-piperazinomethylene-10,11-ethylenedioxy-20(S)-camptothecin (Compound 30)

A solution of 7-chloromethyl-10,11-ethylenedioxy-20(S)-camptothecin (5.2 mg, 11.4×10⁻³ mmol) in anhydrous DMSO (200 uL) was added dropwise to a 0° C. solution of 1-(*a, a, a*-trifluoro-m-tolyl)-piperazine (10 uL, 53×10⁻³ mmol) in anhydrous toluene (500 uL). The dark brown mixture was stirred at 0° C. for 90 min, and allowed to warm to ambient temperature. The solvent was removed with a rotary evaporator and further pumping under high vacuum to leave the crude product, which was purified by silica gel chromatography (eluting with 100% ethyl acetate followed by 6:5:1 ethyl acetate/chloroform/methanol) to afford 3.7 mg (50% yield) of the product as a pale yellow solid residue. ¹H NMR (200 MHz, DMSO-d₆): δ 0.90 (t, 3H, J=7); 1.95 (q, 2H, J=7); 2.60–2.70 (m, 4H); 3.20–3.30 (m, 4H); 4.10 (s, 2H); 4.50 (s, 4H); 5.30 (s, 2H); 5.45 (s, 2H); 6.55 (s, 1H); 7.40 (t, 1H, J=7); 7.60 (s, 1H); 7.85 (s, 1H). Nominal mass spectrum (M+1): 649.

EXAMPLE 31

7-(2-Methoxyphenyl-piperazino)methylene-10,11-ethylenedioxy-20(S)-camptothecin (Compound 31)

To a 0° C. solution of 2-methoxyphenylpiperazine (17.9 uL, 102×10⁻³ mmol) in anhydrous toluene (1 mL) at 0° C. was added a solution of 7-chloromethyl-10, 11-ethylenedioxy-20(S)-camptothecin (10 mg, 22×10⁻³ mmol) in DMSO (200 uL). The dark mixture was stirred at 0° C. for 10 min, and allowed to warm to ambient temperature and stirred for 3 h. The reaction mixture was concentrated with a rotary evaporator and the residual solvent was removed by pumping under high vacuum to afford the crude product. Purification by silica gel chromatography (eluting with 1:1 hexane/ethylacetate followed by 6:5:1 ethylacetate/chloroform/methanol) afforded 3.4 mg (25% yield) of the product as a yellow solid. ¹H NMR (200 MHz, CDCl₃): δ 1.05 (t, 3H, J=7); 1.90 (m, 2H); 2.75 (bs, 4H); 3.10 (bs, 4H); 3.75 (s, 1H); 3.85 (s, 3H); 4.01 (bs, 2H); 5.35 (s, 1H); 5.30 (d, 1H, J=18); 5.35 (s, 1H); 5.75 (d,

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1H, J=18); 6.80-7.00 (m, 4H); 7.60 (s, 1H); 7.65 (s, 1H); 7.75 (s, 1H).

Nominal mass spectrum (M+1): Calcd.: 611. Found: 611

EXAMPLE 32

7-Phenylpiperazinomethylene-10,11-ethylenedioxy-20(S)-camptothecin (Compound 32)

To a 0° C. solution of phenylpiperazine (15.6 uL, 102×10^{-3} mmol) in anhydrous toluene (1 mL) was added a solution of 7-chloromethyl-10,11-ethylenedioxy-20(S)-camptothecin (10.6 mg, 22×10^{-3} mmol) in DMSO (300 uL). The dark mixture was stirred at 0° C. for 10 min, and allowed to warm to ambient temperature and stirred for 3 h. The mixture was concentrated with a rotary evaporator, and the residual solvent was further removed by pumping under high vacuum to afford the crude product as a dark residue. Purification by silica gel chromatography (eluting with 1:1 hexane/ethyl acetate followed by 6:5:1 ethyl acetate/chloroform/methanol) afforded 3.6 mg (30% yield) of the product as a yellow solid residue. ¹H NMR (200 MHz, CDCl₃): δ 1.00 (t, 3H, J=7); 1.90 (m, 2H); 2.75 (bs, 4H); 3.20 (bs, 4H); 3.75 (s, 1H); 4.05 (s, 2H); 4.45 (bs, 4H); 5.35 (s, 2H); 5.30 (d, 1H, J=18); 5.35 (s, 2H); 5.75 (d, 1H); 6.80-7.00 (m, 3H); 7.20-7.35 (m, 2H); 7.60 (s, 1H); 7.65 (s, 1H); 7.80 (s, 1H). Nominal mass spectrum (M+1): 581.

EXAMPLE 33

2'-Amino-4',5'-methylenedioxy-2-dimethylaminoacetophenone

2'-Acetylaminomethylene-2-chloroacetophenone, prepared in Example 1, part (B), is reacted with an excess of dimethylamine under similar conditions as taught in Example 1, part (H), to yield 2'-acetylaminomethylene-2-dimethylaminoacetophenone which is then deprotected by the procedure of Example 1, part (E), to yield 2'-amino-4',5'-methylenedioxy-2-dimethylaminoacetophenone.

Nominal Mass Spectrum M+1: Calcd.: 223. Found: 223.

EXAMPLES 34-38

The following compounds of formula (I) are prepared by the procedure taught in Scheme I or Scheme IA, in an analogous manner to Examples 1-22, using the appropriate intermediate compounds of formulas (II), (III), (IV) and (V).

34: 7-(Methyl-2-methoxyethylaminomethylene)-10,11-methylenedioxy-20(R,S)-camptothecin,

35: 7-Cyclohexylaminomethylene-10,11-methylenedioxy-20(R)-camptothecin,

36: 7-(2-Butenyl)aminomethylene-10,11-methylenedioxy-20(R,S)-camptothecin,

37: 7-Cyclohexylmethylaminomethylene-10,11-ethylenedioxy-20(R)-camptothecin and

38: 7-Thiazolidinomethylene-10,11-methylenedioxy-20(R,S)-camptothecin.

EXAMPLE 39

Pharmaceutical Formulations

5,342,947
28

-continued

(A) Transdermal System	
Ingredients	Amount
Silicone fluid	450.0 mg
Colloidal silicone dioxide	25.0 mg

The silicone fluid and active compound are mixed together and the colloidal silicone dioxide is reacted with to increase viscosity. The material is then dosed into a subsequently heat sealed polymeric laminate comprised of the following: polyester release liner, skin contact adhesive composed of silicone or acrylic polymers, a control membrane which is a polyolefin (e.g. polyethylene), polyvinyl acetate or polyurethane, and an impermeable backing membrane made of a polyester multilaminated. The system described is a 10 sq. cm patch.

(B) Oral Tablet	
Ingredients	Amount
Active compound	200.0 mg
Starch	20.0 mg
Magnesium Stearate	1.0 mg

The active compound and the starch are granulated with water and dried. Magnesium stearate is added to the dried granules and the mixture is thoroughly blended. The blended mixture is compressed into a tablet.

(C) Suppository	
Ingredients	Amount
Active compound	150.0 mg
Theobromine sodium salicylate	250.0 mg
Witepsol S55	1725.0 mg

The inactive ingredients are mixed and melted. The active compound is then distributed in the molten mixture, poured into molds and allowed to cool.

(D) Injection	
Ingredients	Amount
Active Compound	20.0 mg
Buffering Agents	q.s.
Propylene glycol	0.4
Water for injection	0.6 mL

The active compound and buffering agents are dissolved in the propylene glycol at about 50° C. The water for injection is then added with stirring and the resulting solution is filtered, filled into an ampule, sealed and sterilized by autoclaving.

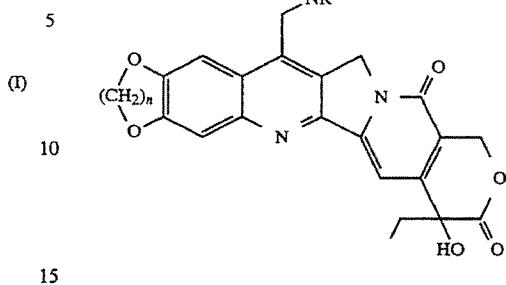
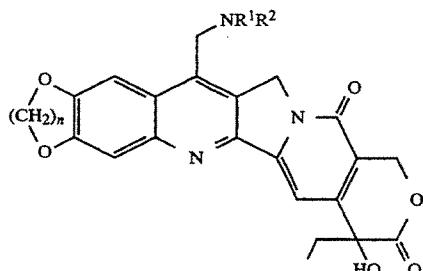
(E) Capsule	
Ingredients	Amount
Active Compound	200.0 mg
Lactose	450.0 mg
Magnesium stearate	5.0 mg

The finely ground active compound is mixed with the lactose and stearate and packed into a gelatin capsule.

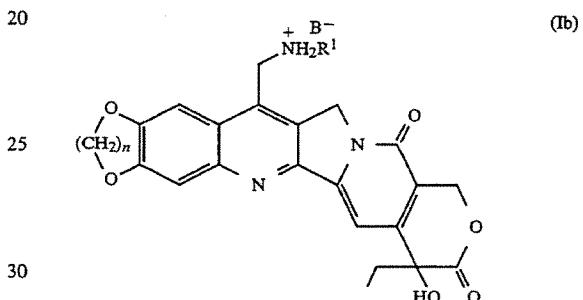
We claim:

1. A method of preparing a compound of formula (I),

(A) Transdermal System	
Ingredients	Amount
Active compound	600.0 mg



wherein Hal represents fluoro, chloro, bromo or iodo, with hydrochloric or sulfuric acid to yield a compound of formula (1b);



wherein:

n represents the integer 1 or 2;

R¹ represents independently, hydrogen, lower alkyl,

30

(C₃₋₇)cycloalkyl, (C₃₋₇)cycloalkyl lower alkyl,

35

lower alkenyl, hydroxy lower alkyl or lower alk-

oxy lower alkyl; and

R² represents hydrogen, comprising:

a) treating a compound of formula (IVb),

wherein B- is a hydrochloride or sulfuric acid anion;

b) treatment of a compound formula (1b), with or without isolation, with about one equivalent of an alkali metal hydroxide or alkali metal carbonate.

2. The method of claim 1 wherein n is 1 and R¹ is

40 hydrogen.

3. The method of claim 1 wherein n is 2 and R¹ is

hydrogen.

4. The method of claim 1 wherein said alkali metal

hydroxide is sodium hydroxide.

45 5. The method of claim 1 wherein said alkali metal

carbonate is potassium carbonate.

* * * * *

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UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,342,947

Page 1 of 2

DATED : August 30, 1994

INVENTOR(S) : Lackey et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 11, line 43, change "Theodore w. Greene" to --Theodore W. Greene--.

Column 11, lines 58-59, change "an aralkyl groups" to --an aralkyl group--.

Column 12, line 10, change "by carried out" to --be carried out".

Column 12, line 25, change "may also be used of the introduction" to --may also be used for the introduction--.

Column 12, line 43, change "Scheme I or Scheme I, be purified" to-Scheme I or Scheme IA may be purified--.

Column 14, line 55, change "other endocrine gland" to --other endocrine glands--.

Column 18, line 3, change "5'(R,S)-1,5-Dioxo-(5'-ethyl-5'hydroxy-2'H, 5'H,6'H,6-oxopyrano" to--5'(R,S)-1,5-Dioxo-(5'-ethyl-5'hydroxy-2'H, 5'H,6'H-6-oxopyrano--.

Column 19, line 56, change "Found 464,1833" to --Found 464.1833--.

Column 20, line 12, change "in place of" to --is used in place of--,

Column 21, line 54, change "prepared according Exam-" to --prepared according to Exam---.

Column 24, line 21, change "Diethyl either" to --Diethyl ether--.

Column 24, line 24, change "(300 MHz, DMSO-⁶)" --(300 MHz, DMSO-d⁶)--.

Column 24, line 27, change "found MH+=291 m/z" to --Found MH+=291 m/z--.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,342,947

Page 2 of 2

DATED : August 30, 1994

INVENTOR(S) : Lackey et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 25, line 16, change "the volatiles are removed" to --The volatiles are removed--.

Column 25, line 22, change "mp>300°C" to --Mp>300°C--,

Column 25, lines 39, change "\$0.90. (t,3H)" to --\$0.90 (t,3H)--.

Column 26, line 42, change "NMR(200MHz, DMSO-d₆); \$0.90" to --NMR(200MHz, DMSO-d₆): \$0.90--.

Column 27, line 39, change "which is turn is deprotected by" to --which in turn is deprotected by--.

Signed and Sealed this
Sixteenth Day of May, 1995

Attest:



BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

EXHIBIT E

Growth, morphology, and serial transplantation of anaplastic human gliomas in athymic mice

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Keywords: human gliomas, athymic mice, GFAP

Abstract

Sixteen of 17 anaplastic human gliomas (AHGs) transplanted into athymic mice produced progressively growing subcutaneous nodules at the site of implantation. Thirty-four of 68 animals (50%) receiving transplanted tissue developed 500 m³ tumors in 24 to 364 days. Fourteen AHG were passed to a second animal generation, and 11 showed continued growth. Eight of these were serially passed, with one reaching a sixth animal generation, four reaching a fifth, and a three third. Once growth occurred in a second animal generation, no AHGs were lost in subsequent passages because of failure to grow. Of 234 animals receiving tumors beyond the first animal generation, 189 (80.8%) developed tumors. Average doubling times of the exponentially growing tumors in serial passage ranged from 3.0 to 19.1 days. This growth rate tended to increase and stabilize in early animal passages. The tumors growing in animals contained cell types which were present in the original human tumors, including fibrillary and protoplasmic astrocytes, small anaplastic cells, gemistocytes, anaplastic spindle cells, and multinucleate giant cells. Glial fibrillary acidic protein (GFAP) was detected in 15 of 17 biopsy specimens and in 12 of 14 AHGs in animals. These data illustrate the value of the athymic mouse system for the investigation of human brain tumors by demonstrating a high rate of successful transplantation, quantitative growth data on serially passed tumors, and morphological and immunochemical resemblance of the tumors in mice to the original human tumors.

Introduction

The athymic mouse has proven to be a reliable host for the growth of some malignant human tumors and offers the prospect of characterization of human neoplastic cells in an *in vivo* setting(1). The value of this system would be strengthened by a high rate of successful transplantation and by the maintenance of representative characteristics of individual tumors. This is particularly relevant for anaplastic gliomas, a biologically heterogeneous group of tumors in which experimental data have been derived largely from diverse animal models(2, 3). We have achieved a high rate of successful subcutaneous transplantation of a series of AHGs into athymic mice, and we have evaluated the morphol-

ogy of the tumors, their expression of a biochemical differentiation marker of astrocytes, and their growth patterns in serial transplantation.

Materials and methods

Animals

Animals were adult athymic BALB/c mice bred at Duke University from an original stock obtained from Sprague-Dawley (Madison, Wisconsin). The animals were maintained under pathogen-free conditions, using sterilized plastic cages covered with polyester bacterial filter tops and were fed sterilized, fat-enriched food with water adjusted to pH 2.3-3.0.

Tumor transplantation

Tumor specimens were obtained directly from the operating room from patients undergoing resections of malignant brain tumors. A representative sample of tumor was fixed in 10% buffered formalin for histological analysis. The remaining tumor was weighed, mechanically minced, and injected subcutaneously into the right flank of the animals in a volume of less than 1.0 ml using a 16-gauge needle. All procedures were performed under sterile conditions.

Morphology of human tumors

All hematoxylin-and eosin-stained (H&E) slides from each patient's surgical procedure were coded, reviewed, and classified according to the World Health Organization classification for brain tumors (4) by one of us (SHB). Selected sections were also stained with Masson trichrome and Wilder reticulin stains.

Tumor growth and passage

Tumors were measured once or twice weekly with calipers, and the volumes were calculated according to the formula $a^2 \times b/2$, where a = width and b = length (5, 6). For tumor passage, the animals were killed by cervical dislocation, tumors were removed under sterile conditions, and, after appropriate material was obtained for histological analysis, the tumors were passed in a modified tissue press through 30/40 mesh cytosieves. Volumes of 50–200 μ d of this processed tissue were then implanted into the right flank of recipient animals using a 20-gauge needle and a Hamilton syringe.

Serially passed tumors were followed until a volume of at least 1 000 mm³ was achieved. Volume doubling times were calculated from sequential measurements once exponential growth began. A volume of 500 mm³ was taken as a measure of successful growth as volumes fluctuated somewhat below this level, progressive growth always occurred once this volume was achieved, and 500 mm³ was on the linear portion of the growth curve in all instances.

Morphology of tumors growing in mice

Portions of subcutaneous lesions from all mice that died spontaneously or were killed were fixed for at least 48 hr in 10% buffered formalin, paraffin embedded, and 5–7 μ sections were stained with H&E. These slides were coded and evaluated for the presence or absence of neoplastic cells. The predominant cellular morphology seen in each lesion was recorded. Stellate and bipolar cells with slender cytoplasmic processes which resembled cell types seen in human astrocytic neoplasms were termed 'fibrillary astrocytes.' Polygonal cells with round nuclei and pink or clear cytoplasm were termed 'protoplasmic astrocytes.' Plump, round cells with abundant eosinophilic cytoplasm were termed 'gemistocytes.' Small round or fusiform cells with scant cytoplasm were termed 'small anaplastic cells.' Large, densely packed, spindle-shaped cells were called 'anaplastic spindle cells.' Large, bizarre multinucleate cells were considered 'multinucleated giant cells.'

Immunohistochemical staining of tumor tissue for GFAP

The tumors were stained for GFAP (7, 8) by the peroxidase anti-peroxidase technique (9) using rabbit anti-human GFAP serum kindly supplied by Dr. Lawrence F. Eng. This technique was applied to formalin-fixed, paraffin-embedded tissue as described in detail by Jones et al. (10). The slides were considered GFAP positive when cells judged neoplastic on morphological grounds contained reaction product.

Results

Morphology of the original human biopsies

Fifteen of the 17 biopsies possessed the typical features of glioblastoma multiforme (GBM). They contained various combinations of fibrillary, protoplasmic, and gemistocytic astrocytes, round or fusiform anaplastic cells, and bizarre multinucleate giant cells (Fig. 1A). Necrosis with or without pseudopalisading, increased vasculature, endothelial proliferation, and mitotic figures were common. One of 17 cases (N-137) was composed of

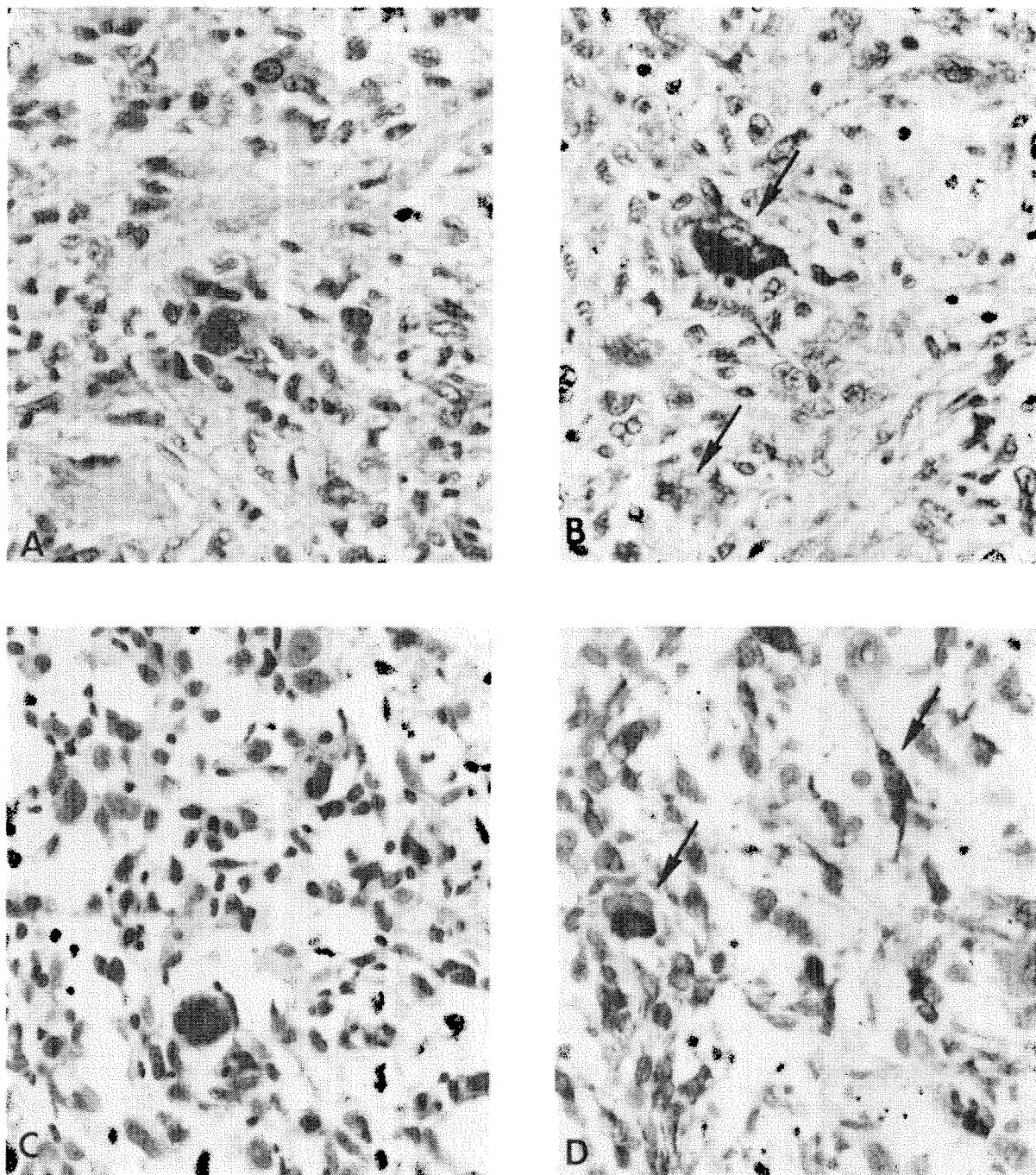


Fig. 1. (A) The original human biopsy in case N-391 is composed of anaplastic fibrillary and protoplasmic astrocytes, with numerous bizarre multinucleate giant cells (H&E $\times 400$); (B) This same field stained with an immunoperoxidase procedure for demonstrating glial fibrillary acidic protein (GFAP) shows cytoplasmic staining (arrows) in a variety of cellular configurations (GFAP $\times 400$); (C) The first passage of N-391 tumor in the mouse shows a mixture of cell types (H&E $\times 400$); (D) Numerous cells are positive for GFAP (arrows) in the first passage tumor in the mouse (GFAP $\times 400$).

Table 1. Growth of 17 anaplastic human gliomas after initial transplantation into athymic mice.

Tumor	No. of animal tumors reaching 500 mm ³	Amount of tumor transplanted per animal (mg)	(Median, Range)	
			Days from transplantation to 500 mm ³ tumor volume	Tumor volume doubling time (days)
N-90/94	3/12	200	161, 157-277	10.7, 5.7-18.8
N-112/115	8/12	190	139, 65-225	14.5, 8.2-22.0
N-120	3/3	260	219, 156-253	17.8, 6.1-30.8
N-132	5/5	80	108, 99-123	20.6, 8.6-27.8
N-137	0/3*	85	-	-
N-142	0/2**	460	-	-
N-155	0/2***	-	-	-
N-175	0/5†	-	-	-
N-183	3/4	-	327, 320-362	18.1, 12.7-18.1
N-241	4/4	-	119, 112-131	6.9, 5.0-7.7
N-292/294	2/8	-	170, 104-236	7.0, 4.0-10.1
N-338	1/2	-	89	30.1
N-341	0/1++	-	-	-
N-350	1/1	-	43	7.8
N-391	2/2	-	42, 24-60	11.4, 9.1-13.6
N-456	1/1	2900	63	9.8
N-457	1/1	1300	0+++	12.5

NA Not available.

* One of these three tumors had reached 88 mm³ on day 156 and was passed to a second animal generation.** One of these two tumors had reached 418 mm³ on day 265 and was passed to a second animal generation.*** One of these two tumors had reached 196 mm³ on day 415 when the animal was killed.† One of these five tumors had reached 405 mm³ on day 76 and was passed to a second animal generation.

++ This tumor was treated with 0.125% trypsin for 10 min prior to implantation into the animal. No tumor was evident 165 days after transplantation when the animal was killed.

+++ Smallest volume = 642 mm³ on day 42.

anaplastic astrocytes, but lacked endothelial proliferation, necrosis, and mitotic activity, and it was considered an anaplastic astrocytoma. One case (N-175) was a re-excision of a gliosarcoma. It contained abundant necrosis and small areas of anaplastic spindle cells consistent with a sarcoma.

Growth after initial transplantation

Tissue from the 17 AHG was transplanted into 68 athymic mice in the initial animal passage (Table 1). Each of four AHGs was transplanted into single animals, and 13 were divided into equal portions and transplanted into two or more animals. The amount transplanted ranged from 80-2900 mg per animal. As the series progressed, we transplanted larger amounts of tumor into fewer individual animals. Two specimens (N-175 and N-457) were obtained from patients undergoing second resections after both radiation and chemotherapy had

been administered; in one of these cases (N-457), tissue had also been obtained from the patient's initial resection (N-350).

Sixteen of 17 AHGs (94%) showed progressive growth in the initial passage in at least one of the recipient animals. Twelve produced at least one subcutaneous tumor of greater than 500 m³. Three (N-137, N-142, and N-175) formed small nodules which were passed before reaching 500 mm³. One (N-155) grew in one animal, but the animal was killed because of illness when the tumor was 196 mm³. One AHG (N-341) had shown no evidence of growth when the animal was killed 165 days after transplantation. This tumor was the only specimen exposed to trypsin prior to transplantation. Tumors grew as circumscribed, lobulated masses which sometimes invaded surrounding soft-tissue structures, but the body cavities were not invaded and no metastases were observed. They characteristically regressed for variable periods after transplan-

Table 2. Morphological and biochemical characterization of anaplastic human gliomas transplanted into athymic mice.

	Human tumor	Animal tumor		GFAP
		GFAP	Predominant cell type	
N-90/94	Glioblastoma multiforme (GBM)	+	Small anaplastic cells	+
N-112/115*	GBM	+	Fibrillary astrocytes	+
N-120	GBM	+	Protoplasmic astrocytes	ND
N-132	GBM	+	Anaplastic spindle cells	+
N-137	Anaplastic astrocytoma	+	Fibrillary astrocytes**	ND
N-142	GBM	+	Fibrillary astrocytes	+
N-155	GBM	+	Fibrillary and protoplasmic astrocytes	-
N-175	Gliosarcoma	-	Anaplastic spindle cells	-
N-183	GBM	+	Fibrillary astrocytes Gemistocytes	+
N-241	GBM	+	Small anaplastic fusiform cells	+
N-292/294	GBM	+	Fibrillary astrocytes Gemistocytes Small anaplastic cells	+
N-338	GBM	+	Fibrillary astrocytes	+
N-341	GBM	+	NA	NA
N-350	GBM	+	Fibrillary astrocytes**	+
N-391	GBM	+	Fibrillary astrocytes Small anaplastic cells Multinucleate giant cells	+
N-456	GBM	+	Fibrillary astrocytes	+
N-457	GBM	UI	Fibrillary astrocytes	+

NA Not applicable.

ND Not done.

UI Uninterpretable due to necrosis.

* In an accompanying paper (10), this tumor was treated as three separate lines (N-112, N-114, N-115) for morphological analysis.

** Nests of tumor in much fibrous tissue.

tion, leaving either no palpable tumor or a small mass which fluctuated slightly in size until exponential growth began.

Thirty-four of the 68 animals (50%) receiving directly transplanted tumors developed tumor nodules of at least 500 mm³. Latency from transplantation to 500 mm³ tumor volume ranged from 24 to 364 days in 33 animals (Table 1). In one animal receiving 1 300 mg of tumor (N-457), the smallest tumor volume was 648 mm³ on day 42, after which progressive tumor growth occurred. Doubling times of these 34 mouse-borne tumors from 12 AHGs ranged from 4.0 to 30.8 days. There was no significant correlation between latency to 500 mm³ volume and subsequent doubling time ($r = 0.23$, $p = 0.19$).

Morphology of first passage tumors in mice

The predominant cellular morphology of tumors growing in the initial transplant generation is shown in Table 2. Nine cases showed only fibrillary and protoplasmic astrocytes. Four showed a homogeneous population of round, fusiform, or spindle-shaped anaplastic cells. Three showed a mixture of cell types (Fig. 1C), and no tumor was available for examination in one case.

Growth in serial transplantation

Fourteen of the 16 AHGs which showed progressive growth in the first animal passage were passed into a second generation. Eleven of these 14 showed progressive growth with animal tumors reaching 500 mm³, while no tumors had appeared in three

Table 3. Growth of serially transplanted anaplastic human gliomas in athymic mice.

Tumor	Animal passage level	No. of animal tumors reaching 500 mm ³ /No. of animals receiving tumor	Mean tumor volume doubling times (Days ± S.D.)
N-90/94	2	4/6	6.4 ± 1.3
	3	14/14	6.1 ± 2.1
	4	4/4	5.7 ± 0.9
	5	4/4	8.7 ± 0.7
	2	17/22	15.5 ± 8.3
N-112/115	3	25/34	8.5 ± 4.3
	4	17/23	7.2 ± 5.0
	5	9/9	5.0 ± 1.1
	2	5/8	8.6 ± 2.4
N-120	3	4/10	8.8 ± 2.9
N-132	2	5/12	15.2 ± 6.2
	3	1/5	8.8
N-175	2	2/2	8/2 ± 0.1
	3	4/4	3/7 ± 0.4
	4	5/5	3.0 ± 1.6
	5	2/2	4.0 ± 0.7
	6	1/1	3.3
	2	4/4	3.6 ± 0.6
N-241	3	5/5	3.2 ± 1.0
	4	11/11	4.0 ± 0.8
	5	4/4	5.0 ± 1.3
	2	1/2	11.4
N-294	2	1/1	9.6
N-350	2	3/3	8.9 ± 0.6
	3	2/2	19.1 ± 10.1
N-391	2	2/2	6.0 ± 1.7
	3	5/5	6.0 ± 1.1
	4	14/16	7.8 ± 2.1
	5	10/10	3.3 ± 0.5
N-456	2	4/4	7.1 ± 1.5
N-457	2	4/4	

(N-338, N-142, and N-137) when the animals were killed 31, 156, and 274 days after passage. One AHG (N-175) was serially passed to a sixth animal generation, four (N-90/94, N-112/115, N-241, and N-456) reached a fifth generation, three (N-120, N-132, and N-391) reached a third, and three (N-292/294, N-350, and N-457) were not passed beyond a second (Table 3). Tumors were frozen for storage at each animal passage level. Once growth occurred in a second animal generation, no AHGs were lost because of failure to grow in serial transplantation.

Of 234 animals receiving tumor from the second through the sixth passage levels 500 mm³ tumors were achieved in 189 (80.8%, Table 3). Average tumor volume doubling times ranged from 3.0 days (N-175, fourth passage) to 19.1 days (N-391, third passage). Within individual AHGs there were ten-

dencies for doubling times to decrease (Tables 1 and 3) and growth patterns to stabilize (Fig. 2) in early animal passages.

Morphology of serially passaged mouse tumors

With serial passage, tumors frequently became more densely cellular, but individual cellular morphology remained unchanged in four of five tumors reaching at least the fourth animal passage level. In one case (N-456) a population of anaplastic spindle cells gradually became dominant. The morphological evolution of these tumors in serial animal passage is discussed in greater detail in a companion paper (10).

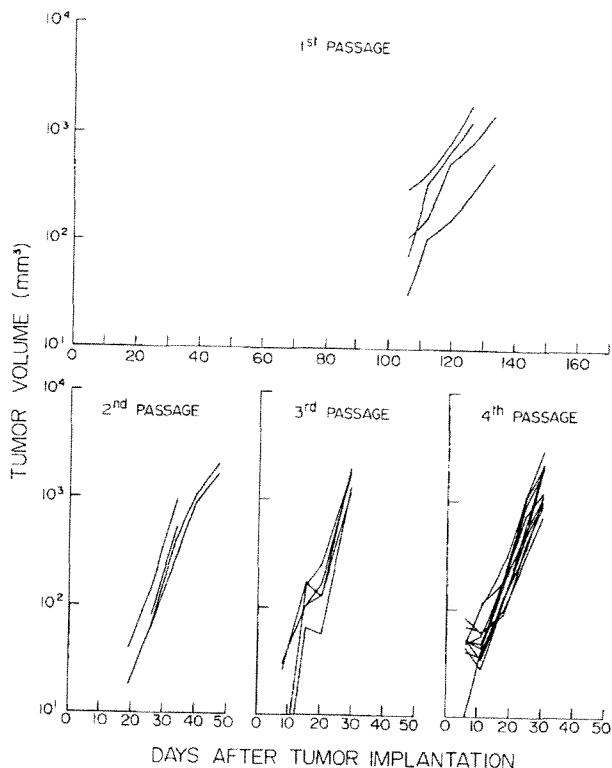


Fig. 2. Graphs illustrate the growth of human tumor N-241 in serial transplantation in athymic mice. In serial passage latency declined and growth patterns stabilized.

GFAP

Fifteen of 17 AHGs contained GFAP in the original biopsy material (Fig. 1B, Table 2). Animal tumors in early passage from 14 of these AHGs were also examined, and 12 were positive (Fig. 1D). One tumor which was positive in the biopsy was negative in the animal (N-155). The other negative animal tumor was an anaplastic spindle tumor from a patient with gliosarcoma (N-175). Two AHGs which were positive in the biopsy (N-120 and N-137) were not examined in the animal. Changes in the pattern of GFAP expression in serial passage are discussed by Jones et al. (10).

Discussion

Growth of transplanted tumors in athymic mice

The theoretical advantages of the athymic mouse for the investigation of human tumors center on our

ability to study proliferating neoplastic cells of human origin in an animal system. However, not all human tumors grow after transplantation into these animals. Melanomas (1, 11), lung cancers (12, 13), and malignant tumors of the gastrointestinal tract (12, 14) have been the most readily established tumors in this system, while tumors of the hematopoietic system and endocrine-dependent tumors have been more difficult to grow (11, 12, 14). In the largest series, from 14.6% (12) to 56% (1) of transplanted tumors have grown in athymic mice, depending primarily on the tumors under consideration, but also on the techniques and definitions utilized. Irradiation of the recipient animals has been reported to increase the rate of successful tumor transplantation (15).

Primary brain tumors have also been reported to grow successfully in these animals. Rana et al. (16), Reid et al. (12), and Wara et al. (17) have reported subcutaneous tumor growth in individual human GBMs transplanted into athymic mice. Shapiro et al. (18) successfully transplanted seven of seven anaplastic brain tumors subcutaneously and six of seven intracerebrally. The two serially transplanted tumors they reported were morphologically both 'gliosarcomas'. In a subsequent report these investigators described 19 successful subcutaneous transplantations of human gliomas, but neither tumor morphology nor growth data were provided in detail and a number of animals were lost due to murine hepatitis (19).

In this series, 16 of 17 (94%) AHGs showed progressive growth after subcutaneous transplantation. The single tumor which did not grow had been exposed to trypsin prior to transplantation, and this may have affected its ability to grow. The high rate of successful 'takes' with AHGs is unexplained; specifically, there are insufficient data to permit conclusions about the relative importance of technical and biological factors. However, the health of our animal colony has permitted a period of observation which exceeds that reported in most series. A number of AHGs required over six months before growth was apparent, and in one case (N-183, Table 1) over 300 days elapsed before progressive growth appeared in three of four animals in which tumor had been implanted. These three tumors developed at the site of implantation were grossly similar to the tumors growing in other animals, were cytologically compatible with anaplastic glial cells, and

contained GFAP (Table 2). We presume that a small number of tumor cells survived the transplantation and required a prolonged period of time, even with exponential growth, to become apparent. Other factors, as discussed by Reid et al. (12), also undoubtedly influence the growth of human tumors in immunologically incompetent animals, including the ability of the animal to vascularize the tumor, the hormone-dependency of the tumor, and residual immunologic activity of the recipient animals, but no attempt will be made to analyze those factors here.

Achievement of a tumor volume of 500 mm³ was considered evidence of successful growth as tumor volumes fluctuated below this level and 500 mm³ was on the linear portion of the tumor growth curve in every instance. The time required to reach a volume of 500 mm³ after transplantation was highly variable, ranging from 24 to 362 days in 33 animals from 11 AHGs, possibly because individual animals received larger initial tumor volumes in some cases. The correlation of time to 500 mm³ tumor volume and subsequent doubling times of the respective progressively growing tumors in the first animal passage was poor, indicating the relatively minor influence of the intrinsic growth rate of these tumors on this variable initial latency period.

Morphology of tumors in mice

The subcutaneous tumors growing in mice contained morphologic cell types which could be recognized in the parent human tumor, but there was a tendency for the mouse-borne tumors to be more homogeneous. Thirteen of 16 tumors in the first animal passage contained a uniform population of either fibrillary and protoplasmic astrocytes or anaplastic cells, while three animal tumors (N-183, N-292/294, and N-391) contained a mixture of cell types. The histologic pattern was not changed in serial passage in four of five tumors passed beyond a third animal generation, although there was a tendency for tumors to become more densely cellular. The remaining tumor became progressively more 'sarcomatous'.

Morphological similarity to the parent tumor has been reported with most tumors successfully transplanted into athymic mice. Povlsen et al. (20) reported a strong correlation between the human and mouse tumors, noting particularly that 'special

properties . . . such as mucin production and formation of melanin granules are preserved.'

Giovanella et al. (1) described similar histologic identity in a variety of carcinomas. Ikeuchi et al. (21) found consistent preservation of cytological features of a series of transplanted lung tumors and included one example of a tumor which produced anti-diuretic hormone, adrenocorticotrophic hormone, melanocyte-stimulating hormone, and calcitonin in the mouse. Some authors have found that some of the differentiated features of the human tumors have been lost in the animal (14), a change which has been attributed to either 'dedifferentiation' or selection for 'the most poorly differentiated parts of the primary tumor.' The transplanted GBMs reported by Rana et al. (16) retained the cytological features of the original tumor, including pleomorphism, palisading, and necrosis, while Wara et al. (17) reported a GBM which became 'more sarcomatous' with successive animal passage. Reid et al. (12) described a grade IV astrocytoma which became more densely cellular in serial passage in the athymic mouse. The present series of 16 transplanted AHGs includes examples similar to each of these previously reported cases. These differing observations concerning the morphology of human gliomas when grown subcutaneously in nude mice are, therefore, not contradictory but represent variable behavior among the individual tumors.

The presence of glial fibrillary acidic protein (GFAP), a specific marker of fibrillary astrocytes (22), is a further indication of the cellular origin of these mouse-borne tumors. Though cytologic identity between the biopsy material examined for GFAP and the bulk of tissue transplanted into the animal could not be assured, all but one AHG which contained GFAP in the biopsy material was also positive in the animal.

Growth of tumors in serial animal passage

Eleven of 14 AHGs passed into a second animal generation produced at least one 500 mm³ tumor. Of the three which did not grow in a second passage, two had been passed from small (< 500 mm³) first passage tumors which were not clearly established, and one (N-338) was observed in the second passage for only 31 days. No other tumors were lost due to failure to grow in serial passage. Overall 80.8% of

animals receiving tumor in serial passage developed progressive tumor growth, and this figure increased to 96.4% (80/83) in the eight most recent tumors, with increasing refinement of our techniques. While some differences in 'take' were apparent among different tumors (e.g., N-132 vs. N-241), we cannot be sure that these differences were due to properties of the tumors rather than to technical factors. Since tumors were passed into a number of animals simultaneously, any technical error in an individual passage would be magnified.

Growth rates, as determined by tumor volume doubling times, tended to stabilize in serial passage. First and second passage tumors tended both to grow somewhat more slowly and to show greater inter-animal variability. There was also a tendency for some AHGs (e.g., N-175 and N-241) to grow more rapidly and with less variability than others, but overall the number of animals was insufficient to allow conclusions about these differences.

Most investigators studying human tumors in the nude mouse have reported relatively constant tumor growth rates in serial animal passage. Povlsen et al. (23) noted variation in growth rate among different tumors but a 'constant' pattern within individual tumors in serial passage, though quantitative data were not provided. Rae-Venter and Reid (24) also found that breast tumors maintained their growth pattern in serial passage. Mattern et al., on the other hand, reported growth acceleration in serial animal passage in a series of lung tumors (25). While Reid et al. (12) reported that 'the growth rate assumed in the first nude mouse host remains constant throughout subsequent passages,' they noted an exception to this in one anaplastic glioma which showed an increasing growth rate in serial passage, an observation they explained by assuming 'selection . . . for the fastest growing tumorigenic cell type.' Shapiro (18) also noted an increasing growth rate in two serially transplanted gliosarcomas in the early animal passages in a pattern similar to that reported here, although he did not comment on inter-animal variability in serial passage.

Human anaplastic gliomas show striking morphological and biological heterogeneity (26-29). It is not known which of the many cellular components of such a tumor are truly neoplastic or whether the different neoplastic cell populations have different growth potentials. Selection in the animal for the most rapidly growing portion of the trans-

planted tumor may explain the patterns of growth in serial animal passages reported here and by others. Alternatively, adaptation to an unfamiliar environment might produce behavioral changes in a neoplasm without altering its fundamental characteristics.

The high rate of successful transplantation demonstrated here indicates that this system is particularly appropriate for the analysis of individual human glial neoplasms, including determinations of their therapeutic sensitivities. Bullard et al. (6) have reported the results of treatment with 1-3, bis (2-chloroethyl)-1-nitrosourea (BCNU) of three human glioma-derived permanent cell lines growing in athymic mice, and Shapiro et al. (18) have treated two human glioma xenografts with a battery of agents including BCNU and procarbazine. Use of a variety of chemotherapeutic agents against a series of transplanted, serially passed human gliomas in athymic mice should provide important information about the variability and patterns of chemosensitivity of these tumors.

Acknowledgement

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EXHIBIT F

In Vivo Behavior of Genetically Engineered Herpes Simplex Viruses R7017 and R7020: Construction and Evaluation in Rodents

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The herpes simplex virus (HSV) recombinant R7017 was constructed from HSV-1 (strain F) by deleting a portion of the thymidine kinase (*tk*) gene and by replacing the sequences representing the internal inverted repeats and adjacent genes in the L component with a fragment of the HSV-2 genome encoding the glycoproteins G, D, I, and a portion of E. In addition, the R7020 recombinant contains an HSV-1 DNA fragment encoding the *tk* gene fused to the $\alpha 4$ gene promoter. The results of studies in mice, guinea pigs, and rabbits were as follows: Both recombinants remained unchanged after nine serial, intracerebral passages in mice; the recombinants could not be differentiated with respect to attenuation in mice injected intracerebrally, in vaginally infected guinea pigs, and in rabbits inoculated on the scarified cornea. Given intradermally or intramuscularly, the recombinants prevented severe infections by virulent challenge viruses, and R7020 established latent infections (at a low frequency) in all species tested, whereas latent R7017 virus was detected in rabbits only.

The objective of an immunization program to protect against infections with herpes simplex virus 1 and 2 (HSV-1 and HSV-2) is, minimally, twofold. The first objective is to reduce the incidence and severity of initial infection with each of these agents. The second and even more important objective is to preclude the colonization of sensory ganglia with these agents, because much of the morbidity associated with HSV infections is the result of reactivation of virus latent in sensory ganglia. *A priori*, the latter objective appears to be particularly difficult to attain inasmuch as colonization of sensory ganglia occurs as a consequence of multiplication of virus during the initial stages of infection at the portal of entry. To protect against replication at the portal of entry and subsequent colonization of ganglia, the level of initial immunity must be quite high, inasmuch as colonization of ganglia would be likely to occur before the host immune system could be expected to

respond to the antigenic mass of the infecting virus. Once in the sensory ganglion, the latent virus becomes shielded from the immune system. The possibility that a vaccine could be effective is nevertheless apparent from studies showing that individuals with prior exposure to HSV-1 usually experience milder first infections with HSV-2 [1] and that reinfections with new strains of the same serotype are infrequent in immunocompetent patients [2], an occurrence indicating that human infections with HSV are followed by at least some protective immunity. Consistent with the notion that to attain protection the level of immunity must be similar to that attained after a natural infection with HSV, it is our expectation that only a live attenuated vaccine is likely to induce the level of immunity necessary to preclude the entrenchment of the virus at the portal of entry into the body [3]. An added advantage of a live attenuated virus vaccine is that it could also serve as a vector for expression of gene-specifying antigens capable of inducing immunity against other infectious agents in humans.

In this paper, we report on the construction and initial biologic characterization of two recombinant viruses designed to function as prototypes of live attenuated HSV vaccines. The objective of the experimental design of the recombinant viruses was to introduce permanent, irreversible, easily identifiable genotypic changes in the HSV genome. The phenotypic effects of these changes were (/) to at-

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tenuate the capacity of HSV to cause disease resulting from neuronal tissue destruction or activation of latent virus, (2) to protect against both HSV-1 and HSV-2 infection without obscuring evidence of post-vaccination HSV-2 infection, and (3) to enable the recombinant virus to serve as a vector for expressing genes specifying protective antigens of other agents infecting humans. To attain these objectives, we deleted 700 base pairs (bp) from the domain of the thymidine kinase (*tk*) gene and 14 500 bp from the region extending from the 3' terminus to the $\alpha 27$ gene in the L (long) component across the internal inverted repeats to the promoter regulatory domain of the $\alpha 4$ gene mapping in the S (short) component. The purpose of the deletions engineered into the recombinant virus genome was twofold: to excise some of the genetic loci responsible for neurovirulence [4] and to create convenient sites and space within the genome for inserting other genes. Into this genetically engineered HSV-1 backbone, we inserted an HSV-2 DNA fragment encoding the HSV-2 glycoproteins D, G, I, and a truncated portion of E to form R7017, and we inserted both this fragment and the *tk* gene to form R7020. The rationale for the insertions stems from the observations that some of the glycoprotein genes contained in that fragment show the maximum divergence between HSV-1 and HSV-2 [5]. Glycoprotein D appears to induce the highest levels of neutralizing antibodies to HSV that are at least in part specific. The *tk* gene was restored to enhance the ability of the virus to multiply efficiently at the site of inoculation and to make it susceptible to antiviral chemotherapy. The gene was modified in such a way as to preclude it from recombining at its natural position.

The biologic properties of the recombinant viruses reported here were tested in three species of laboratory rodents. These species and the routes of inoculation used were primarily selected for their pertinence to the evaluation of genetic stability, virulence, and ability to establish latent infections.

Materials and Methods

Cells and viruses. Vero cells obtained from the ATCC (Rockville, Md) were used to prepare stocks of wild-type viruses, to titrate all stocks of virus, and to isolate virus from biologic specimens. Stocks of recombinant viruses were prepared in human diploid MRC-5 cells (Medical Research Council, London, England) at a multiplicity of 0.01 pfu per cell. The

transfections for the selection of recombinant viruses were done in rabbit skin cells, as previously described [6]. The selections for *tk*⁻ and *tk*⁺ viruses were done in the human 143 *tk*⁻ cell line, as previously described [6].

The origin and properties of HSV-1 strains F and MGH10 and of HSV-2 strain G were reported elsewhere [7, 8]. HSV-1 strain McKrae was a gift from Y. Centifanto-Fitzgerald (Tulane University School of Medicine, New Orleans). HSV-1(MGH10) and HSV-2(G) have been used extensively in our laboratories in challenge infections because of their pathogenicity in mice and in guinea pigs. The McKrae strain was selected for studies in rabbits because the pattern of ocular disease produced by this virus has been well characterized and described in the literature [9]. In our experience, this strain caused severe eye disease without the necessity of scarifying the cornea before inoculation.

DNAs. The restriction endonuclease digestion, cloning, and purification of plasmid and viral DNA were as described [10, 11].

Monoclonal antibodies and assays for expression of HSV-1 and HSV-2 glycoproteins. The production of specific glycoproteins by the recombinant viruses was tested by using the biotin-avidin-enhanced surface immunoassay with monoclonal antibodies H600, H966, H1301, H1379, and H1380 to HSV glycoproteins [12].

Studies in animals. Six-week-old male, inbred BALB/c or outbred OF1 mice and 300–400-g Dunkin-Hartley female guinea pigs were obtained from IFFA-Credo (Les Oncins, France). New Zealand rabbits (2 kg) were purchased from Elevage Scientifique des Dombes (Chatillon sur Chalaronne, France). Randomly sorted groups were maintained for at least a week before inoculation. Rabbits and guinea pigs were free of HSV-neutralizing serum antibodies at the time of the first inoculations. Inoculations by intracerebral or eye routes and blood collections were done while animals were anesthetized with pentobarbitone (Clin Midy Vétérinaire, Saint Jean de la Ruelle, France), ether (for mice), or ketamine (Rhône-Mérieux, Lyon, France; for guinea pigs and rabbits). Terminal bleedings were done while animals were deeply anesthetized with pentobarbitone. Stocks of virus were diluted in PBS supplemented with 0.1% glucose and 1% fetal calf serum (PBS-GS) to the required concentrations, as stated in the Results. The actual titers of the stocks were determined independently in each experiment.

Isolation of infectious virus. Swabs used to collect biologic specimens were kept in PBS-GS at 4°C until plating on Vero cell cultures. Sensory ganglia assayed for the presence of infectious virus were removed at autopsy, homogenized in PBS-GS, and subjected to three cycles of freezing and thawing before they were plated onto cell layers. All specimens were processed within <2 h of the time of collection. To detect infectious virus, we exposed the cell cultures to specimens for 1 h under agitation, then replenished the specimens with maintenance medium 199V (mixture 199 supplemented with 1% newborn calf serum) and incubated them at 37°C (for wild-type viruses) or 34°C (for recombinants). The cultures were examined daily for seven days for the development of CPE.

Assay for latent virus. At least four weeks after inoculation, trigeminal or dorsal root ganglia were aseptically dissected, incubated for four or five days in medium 199V_s (mixture 199 supplemented with 5% calf serum) to allow for the reactivation of latent virus, homogenized, and plated onto Vero cell

layers. As a confirmatory test, most negative cultures were harvested and freeze-thawed on the seventh day of incubation; the resulting suspension was then plated onto fresh Vero cells.

Neutralization tests. Serum-neutralizing antibodies were measured by the micro method [33], on serial twofold dilutions of the serum against 100 TCID₅₀ of either HSV-1(MGH10) or HSV-2(G). Titers were calculated by the Karber method and were expressed as the reciprocal of the highest dilution that reduced the virus engaged in the reaction from 100 to 1 TCID₅₀.

Results

Construction of R7017 and R7020. The recombinant strains R7017 and R7020 used in these studies were derived from HSV-1(F) [7] by using a previously described experimental procedure in which the HSV-1(F) *tk* gene is used as a selectable marker for engineering recombinant strains of large DNA viruses [13, 14]. The recombinants were constructed in

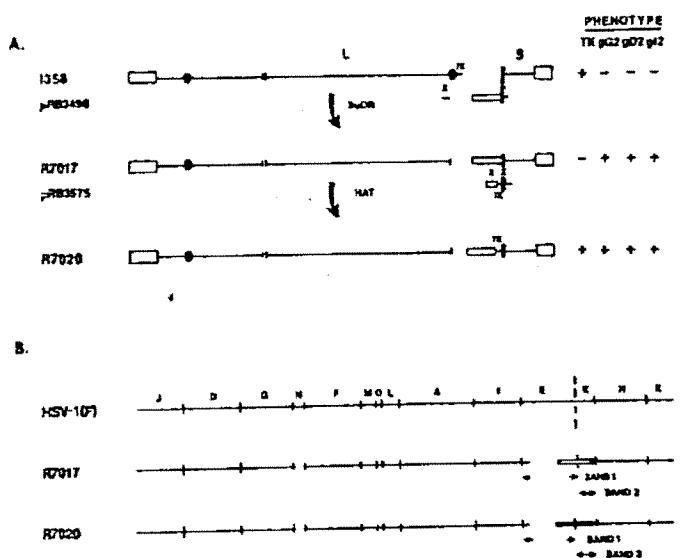


Figure 1. Schematic representation of the structure of the recombinants used. **A**, details of the construction of the recombinants R7017 and R7020. I358, a recombinant virus derived from HSV-1(F) [15], was cotransfected with pRB3498, and the *tk*⁺ recombinant R7017 was selected for its ability to grow in the presence of BuDR (5-bromo-deoxyuridine). The closed circles in the I358 genome indicate reiteration of the *Hind*III O sequences present at the left-hand portion of the genome and duplicated at the junction between the L and S components of the I358 genome. The large open box corresponds to the HSV-2(G) *Hind*III L fragment inserted into the R7017 genome. R7020 was constructed by cotransfection of R7017 with pRB3575 and the subsequent selection of *tk*⁺ virus in HAT (hypoxanthine, aminopterin, thymidine) medium. The respective phenotypes of each of the recombinant viruses are listed. The deletion in the *tk* gene (TK), present in all the recombinant genomes, is indicated by a double line transecting the L component line. **B**, the EcoRI restriction endonuclease maps of HSV-1(F), R7017, and R7020 DNAs. The numbered fragments correspond to the novel bands indicated in figure 3.

several steps determined largely by the desired features. (1) The specific shared properties of the R7017 and R7020 recombinants are a 700-bp deletion in the transcribed noncoding and coding domains of the *tk* gene and a 14.5-kilobase pair deletion spanning the unique and reiterated sequences extending from the 3' terminus of the $\alpha 27$ gene to the promoter domain of the $\alpha 4$ gene. To test whether a recombinant virus with these features would be viable, we constructed an intermediate recombinant virus, designated as R3410, from I358, a previously published recombinant virus derived from HSV-1(F) [15]. In the recombinant I358 genome, most of the internal inverted repeated sequences were replaced by a DNA sequence containing the *tk* gene and a reiterated portion of the *Hind*III O fragment mapping near the left terminus of the HSV-1 DNA (figure 1). As a result of the deletion, the components of the I358 genome were frozen and were no longer inverted relative to each other. The precise borders of the deleted sequences were not, however, known. Moreover, because of the reiteration of the portion of the *Hind*III O fragment, the I358 virus generated defective genomes during replication [16].

To produce the appropriate HSV-1 backbone for the R7017 and R7020 recombinants, we cotransfected intact I358 DNA with excess pRB3410 plasmid DNA, and the *tk*⁻ progeny was selected and analyzed by restriction endonuclease digestion for expected features. Plasmid pRB3410 was constructed by inserting an 1838-bp *Pvu*II-*Bam*HI fragment and a 2300-bp *Bam*HI-*Sac*I fragment into the *Eco*RI and *Hind*III sites, respectively, of pUC12 (figure 2, line 2). The 1838-bp *Pvu*II-*Bam*HI fragment from HSV-1(F) DNA contained the promoter-regulatory domain of the $\alpha 4$ gene and upstream region, including Ori, and a portion of the $\alpha 22$ gene, whereas the 2300-bp *Bam*HI-*Sac*I fragment contained the entire $\alpha 27$ gene. In the recombinational events between the I358 DNA and the homologous sequences of the pRB3410 DNA, all of the sequences located between the flanking HSV-1(F) sequences in the plasmid DNA would be expected to be deleted. A recombinant virus exhibiting these features was selected, tested for viability, and designated as R3410.

(2) A special feature of the pRB3410 plasmid was the presence of additional restriction endonuclease sites between the $\alpha 27$ and $\alpha 4$ promoter-regulatory region for inserting additional DNA sequences into the viral genome. To construct the prototype vaccine strains, we constructed two additional plasmids.

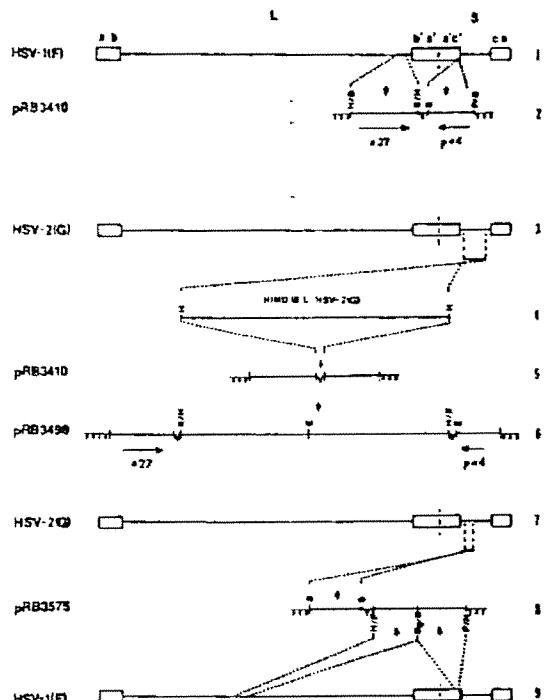


Figure 2. Schematic representations of the DNA sequence arrangements in the plasmid clones constructed for use in these studies. Line 1, sequence arrangement of HSV-1(F) genome. Boxes represent the terminal sequences repeated internally in inverted orientation dividing the genome into the L and S components. Line 2, construction of pRB3410 containing the entire $\alpha 27$ gene and the promoter-regulatory region of the $\alpha 4$ gene and upstream sequences. Line 3, sequence arrangement of the HSV-2(G) genome with the HSV-2(G) *Hind*III L fragment used to construct the pRB3498. Lines 4-6, construction of pRB3498. Line 7, sequence arrangement of the HSV-2(G) genome with the 1766-bp *Hind*III-*Sac*I fragment from the *Hind*III L fragment used to construct pRB3575. Line 8, sequence arrangement of pRB3575. Line 9, sequence arrangement of the HSV-1(F) genome, with sequences contained in the 3627-bp chimeric $\alpha 4$ *tk* gene used to construct pRB3575.

pRB3498 was constructed by inserting the *Hind*III L fragment from HSV-2(G) into a unique *Xba*I site located between the $\alpha 27$ and $\alpha 4$ sequences present in vector pRB3410 (figure 2, lines 4-6). Sequencing data of the *Hind*III L fragment [5] indicate that this fragment contains the HSV-2 counterparts of the HSV-1 genes encoding US2; the HSV protein kinase [17]; US5; the glycoproteins G (gG-2 [12, 18]), D (gD-2 [19]), and I (gI-2 [20]); and a portion of the coding domain for glycoprotein E (gE-2 [19]). The *tk*⁻ prog-

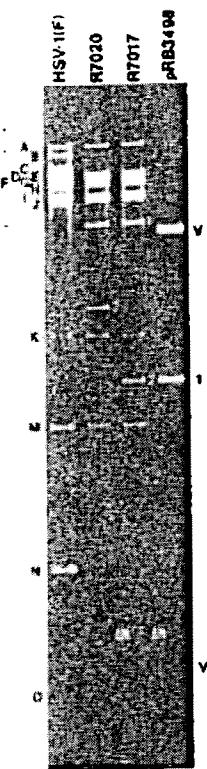


Figure 3. Ethidium bromide-stained, electrophoretically separated EcoRI restriction digests of HSV-1(F), R7017, R7020, and pRB3498. Letters on the left-hand side of the figure correspond to the EcoRI fragments present in HSV-1(F) digests. ΔN identifies the HSV-1(F) EcoRI N band in which 700 bp have been deleted in the two recombinants. The white numbers correspond to the novel bands present in the recombinants and indicated in figure 1, B. The letter V and number 1 on the right-hand side of the figure correspond to the EcoRI fragments of pRB3498. V indicates fragments containing vector sequences, and 1 indicates the EcoRI fragment present in pRB3498 that contains the same sequences and comigrates with the band present in R7017, designated band 2. In R7020, because of the insertion of *tk* sequences, the band designated band 2 shifts to a higher molecular weight and no longer comigrates with the EcoRI fragment present in pRB3498.

eny of the cotransfection of I358 DNA with pRB3498 yielded the recombinant R7017 (figures 1 and 3).

Plasmid pRB3575 was constructed in two steps (figure 2, line 8). In the first step, a 1766-bp *Sac*I-*Hind*III fragment from the HSV-2(G) *Hind*III L fragment that is closest to the $\alpha 4$ promoter-regulatory region in the R7017 recombinant genome was inserted into the *Sac*I site of pUC18 to create plasmid pRB3573. In the second step, a 3627-bp

*Pvu*II fragment containing the promoter-regulatory region of the $\alpha 4$ gene fused to the transcribed domain of the *tk* gene was inserted into the unique *Hind*III site contained within pRB3573, a process resulting in plasmid pRB3575. Plasmid pRB3575 was constructed as a vector with additional restriction sites available between the HSV-2(G) sequences and the $\alpha 4$ -*tk* sequences for inserting foreign DNA into the R7020 genome. Cotransfection of this plasmid DNA with intact R7017 viral DNA and subsequent selection of *tk*⁺ virus yielded R7020 (figures 1 and 3). In this genome, the 5' transcribed noncoding and coding sequences of the *tk* gene were inserted 3' to the HSV-2(G) sequences and were fused to the promoter-regulatory region of the $\alpha 4$ gene (figure 1, A). The genes present in the HSV-2(G) *Hind*III L fragment inserted into the R7017 and R7020 genome are in an inverted orientation with respect to the homologous resident HSV-1(F) genes contained in their natural location in the S component.

Expression of HSV-1 and HSV-2 glycoprotein gene homologs in R7017 and R7020 genomes. For monitoring the genetic stability of the duplicated homologs of the HSV-1 and HSV-2 glycoprotein genes inserted into the R7017 and R7020 recombinant virus genomes, cells infected with these recombinants were tested during plaque purification or serial propagation of virus pools or after isolation from experimental animals by using the biotin-avidin-enhanced surface immunoassay [12]. In all instances the recombinant viruses retained their immunologic reactivity with monoclonal antibodies to gE-1, gE-1+2, gG-1, gG-2, and gD-1.

Biologic properties of R7017 and R7020 in mice. **Experimental system.** Although relatively resistant to HSV infections by peripheral routes, the mouse is highly susceptible to intracerebral inoculation with wild-type HSV-1 or HSV-2 and is therefore suitable for use in studies on viral neurovirulence. For latency studies, we selected the BALB/c strain because of its propensity to become latently infected with HSV [21] and the eye route of infection because it causes higher rates of latent infections than does the ear or the footpad route [22, 23; authors' unpublished data].

Neurovirulence. Titrations were done by injecting 50 μ L of serial 10-fold dilutions of the suspensions of virus intracerebrally to groups of 10 mice. The results, computed by the 2 arc sine p method, are summarized in table 1. They indicate that both R7017 and R7020 require \sim 10 000-fold more virus

Table 1. Biologic properties of R7017 and R7020 recombinant viruses in mice.

Test for	Route of inoculation	Results		
		HSV-1 (F)	R7017	R7020
Virulence*	ic	3.8×10^2	2.7×10^4	2.7×10^4
Latency†	Eye	25/32 (10^4)	0/20 (10^4)	1/20 (10^4)
Exp 1, normal mice				2/19 (10^4)
Exp 2, normal mice			0/20 (10^4)	5/20 (10^4)
Exp 2, immunosuppressed mice				
Genetic stability*	ic	3.4×10^4	3.4×10^4	$>2.0 \times 10^4$
Passage 0		8	4.2×10^4	6.2×10^4
Passage 9				

NOTE. ic, Intracerebrally.

* Data are the no. of pfu that determined the LD₅₀.

† Exp, experiment. Data are the no. of mice in whom latency was established/total no. (no. of pfu per eye).

than does the wild-type HSV-1 parent virus to cause lethal infections in 50% of the animals inoculated intracerebrally.

Recovery of latent virus. Two series of experiments were done. In the first series, the mice were inoculated in the right eye with $\sim 2 \times 10^7$ pfu per eye in 10 mL of either recombinant. Four weeks after inoculation, the mice were killed, and the right trigeminal ganglia were tested for the presence of latent virus. As summarized in table 1, latent virus was recovered from 1 (5%) of 20 mice inoculated with R7020; no latent virus was recovered from the mice inoculated with R7017. For comparison, latency was established in 78% of mice inoculated with only 10^4 pfu of the parent virus, HSV-1(F).

In the second series of experiments the mice were immunosuppressed by three administrations of cyclophosphamide (Endoxan Asta, Laboratoires Lucien, Colombes, France)—one dose of 4 mg/mouse two days before and one day after inoculation and one dose of 2 mg/mouse five days after inoculation with $\sim 2 \times 10^7$ pfu. All mice survived this procedure. As shown in table 1, virus was recovered from the ganglia of five mice inoculated with R7020 but not from ganglia of mice inoculated with R7017.

Genetic stability. The viruses isolated from latently infected animals could not be differentiated from the virus in the inoculum with respect to the restriction digestion patterns of their DNA. Additional tests on the genetic stability of the recombinant viruses were based on previous studies showing that serial passages of virus from mouse brain to cell culture and back to mouse brain select for revertants in virulence markers altered by mutagenesis [24]. In those studies, a revertant was detected in an inter-

typic population of recombinant viruses after six serial passages, whereas a deletion mutant tested in parallel remained unaltered with respect to its ability to grow in the CNS and yielded similar pfu:LD₅₀ ratios even after nine serial passages.

The results of similar studies on R7017 and R7020 are shown in table 1. It is interesting to note that for the wild-type virus, the pfu:LD₅₀ ratio decreased by a factor of 40, a change consistent with the observation that the initial virulence of HSV-1(F) for mice was relatively low compared with that of other wild-type isolates. In contrast, the pfu:LD₅₀ ratios of both recombinants varied by less than fourfold. Moreover, the restriction endonuclease digestion patterns of recombinant virus DNAs at passage 9 could not be differentiated from those of passage 0 (data not shown).

Protection of mice against lethal infection after intracerebral challenge. In this experiment, groups of 60 mice were immunized by the footpad route with 20 μ L of 10-fold dilutions of R7017, R7020, or HSV-1(F), ranging from 10^2 to 10^7 pfu per mouse. Four weeks after immunization, groups of 10 mice were challenged intracerebrally with 3, 30, or 300 LD₅₀ of either HSV-1(MGH10) or HSV-2(G) per mouse. As might be expected, the results (table 2) indicate that more recombinant virus was required to protect against high doses than against low doses of challenge virus and that HSV-1(F) was more efficient than the recombinant viruses in protecting against the high doses of challenge viruses.

Protection of mice against the establishment of latent HSV-1(F) infection. In this test, mice were immunized with 10^7 pfu of R7017 or R7020 by the footpad route and were challenged six weeks later

Table 2. Protection of mice from intracerebral challenge with HSV-1(MGH10) or HSV-2(G) after footpad immunization with R7017 or R7020.

Challenge strain, immunization strain	Protection, at indicated LD ₅₀ *		
	300	30	3
HSV-1(MGH10)			
HSV-1(F)	1.2 × 10 ⁴	3.2 × 10 ²	1.2 × 10 ²
R7017	2.5 × 10 ⁴	5.0 × 10 ⁴	2.0 × 10 ²
R7020	2.5 × 10 ⁴	1.0 × 10 ⁴	6.3 × 10 ²
HSV-2(G)			
HSV-1(F)	7.9 × 10 ²	6.3 × 10 ²	2.0 × 10 ¹
R7017	6.3 × 10 ⁴	4.0 × 10 ⁴	5.0 × 10 ²
R7020	>4.0 × 10 ⁴	6.3 × 10 ⁴	<4.0 × 10 ²

* Data are the no. of pfu that protected 50% of the mice.

with either 10⁴ or 10⁵ pfu of HSV-1(F) by the eye route. Approximately four weeks later the mice were killed, and the trigeminal ganglia were tested for the presence of latent virus. The results (table 3) indicated that immunization with either virus reduced the establishment of latency by the challenge virus. Tested in a separate experiment, HSV-1(F) required a smaller immunizing dose to achieve a similar degree of protection.

The biologic properties of R7017 and R7020 in guinea pigs. Experimental system. In recent years the guinea pig has become a very valuable species, mostly for studying genital infections [25-27]. Vaginal inoculations of wild-type HSV cause typical lesions on the vulvar area, as vesicles or pustules on an inflamed base. Complications of fecal or urinary retention and paralysis of the hind limbs are not uncommon. Death can occur, usually caused by the spreading of the virus to the CNS.

Virulence. In the first series of experiments, guinea pigs were inoculated vaginally with 100 mL of suspension containing 10⁴ or 10⁵ pfu of either R7017 or R7020 virus. The guinea pigs were then monitored daily for the presence of lesions on the external genitalia. At 1, 2, 3, 5, and 7 days after inoculation, vaginal swabs were tested for the presence of infectious virus. Approximately four weeks after inoculation the guinea pigs were killed, and the lumbarosacral ganglia from the fourth lumbar to the third sacral were tested for the presence of latent virus. The results (table 4) indicated that infectious virus was recovered from a small fraction of animals inoculated with 10⁴ pfu and from nearly all animals inoculated with 10⁵ pfu. Shedding of virus was demonstrable up to day 5, and no virus was recov-

Table 3. Recovery of latent virus from trigeminal ganglia of mice immunized with R7017 or R7020 by the footpad route and then challenged with HSV-1(F) by the eye route.

Immunization strain, dose	No. of positive mice/no. of survivors after challenge with		
	Control	10 ⁴ pfu	10 ⁵ pfu
None	ND	13/18	16/16
R7017, 10 ⁵ pfu	0/10	6/20*	14/20†
R7020, 10 ⁵ pfu	0/9	4/19*	8/20‡
HSV-1(F), 10 ⁴ pfu	0/10	ND	5/7

* P < .01 compared with the control group; determined by χ² with Yates's correction.

† P < .05 compared with the control group; determined by χ² with Yates's correction.

‡ P < .001 compared with the control group; determined by χ² with Yates's correction.

ered in tests done seven days after inoculation. The typical lesions seen in animals infected with wild-type virus were not observed in the guinea pigs inoculated with recombinant viruses. Few animals developed either erythematous maculas or small epithelial cracks, and two of the R7017-immunized animals showed one single, small vesicle from which no virus could be recovered. We could not, in fact, ascertain whether such lesions were virus specific or whether they were caused by swabbing of the external genitalia. Latent virus was recovered from one guinea pig inoculated with 10⁵ pfu of R7020 virus. The amount of latent R7020 virus recovered from this guinea pig was significantly less than that routinely recovered from ganglia of guinea pigs infected with wild-type virus. The results of vaginal infections with the wild-type strain HSV-2(G) obtained in a separate experiment, as shown here for comparison (table 4).

In a second series of experiments (table 4), guinea pigs were inoculated on the scarified right cornea with ~10⁴ pfu in 10 μL of either recombinant or with 10⁵ pfu of HSV-1(F). Infection ensued in nearly all animals, and recombinant viruses were recovered from the tear film of the inoculated eyes for three to five days after inoculation; the wild-type virus was shed for one week. The guinea pigs inoculated with the recombinants showed only mild, traumatic lesions for few days, and latent virus was recovered from two of 10 guinea pigs inoculated with R7020. Although R7017 and R7020 caused very similar ocular infections with respect to shedding, symptoms, and lesions, the former was not detected in latency

Table 4. Shedding and symptomatology of wild-type and recombinant HSV inoculated into guinea pigs.

Test measurement	No. positive/total no. tested after								
	Vaginal inoculation with				Eye inoculation with				
	HSV-2(G)		R7017		R7020		HSV-2(F), 10 ³ pfu	R7017, 10 ⁴ pfu	R7020, 10 ⁴ pfu
	10 ³ pfu	10 ⁴ pfu	10 ³ pfu	10 ⁴ pfu	10 ³ pfu	10 ⁴ pfu			
Shedding of virus	5/5	5/5	3/10	8/10	6/10	9/10	10/10	9/10	10/10
Symptoms of lesions	5/5	5/5	0/10	0/10	0/10	0/10	10/10	5/10	4/10
Death	3/5	2/5	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Latent virus	10/16	NT	NT	0/10	NT	1/10	5/10	0/10	2/10

NOTE. NT, not tested.

tests on trigeminal ganglia. In contrast, HSV-1(F) caused more-severe eye diseases, including conjunctivitis, keratitis, and iridocyclitis, in all recipients and established latency in the trigeminal ganglia of 50% of them even though it was used at a lower dose.

Protection of guinea pigs against HSV-2 by immunization with recombinant viruses. In this series of experiments, guinea pigs were immunized with 10⁴ pfu (in 50 µL) of either R7017 or R7020 injected intradermally in the right ear pinna or im in the right thigh. Four weeks after immunization the guinea pigs were challenged vaginally with 10⁵ pfu of HSV-2(G). The guinea pigs were monitored, as described above, for shedding of virus and for lesions. Four weeks after challenge the guinea pigs were killed, and the sets of lumbar and sacral ganglia were each pooled and tested for the presence of latent virus. The salient features of the results (table 5) are that immunization did not preclude shedding of virus or the development of lesions caused by the challenge virus. The lesions were, however, fewer and smaller (figure

4) and healed more rapidly. In addition, the perineal tumefaction seen in immunized animals was greatly reduced or prevented (figure 5). In contrast to the nonimmunized animals, none of the immunized guinea pigs developed paralysis or succumbed to infection. Table 5 also shows the results of a separate experiment showing that HSV-2(G) given intradermally was able to prevent the development of homologous vaginal superinfection.

Biologic properties of R7017 and R7020 in rabbits. Experimental system. The animal of choice according to the first experimenters, the rabbit is still valued for use in studies of ocular infections with HSV. After inoculation by the eye route, the local symptoms and lesions are similar to those seen in human patients, including conjunctivitis, iritis, keratitis, and the development of corneal ulcers. Unlike people, however, rabbits frequently die from encephalitis caused by the dissemination of the infecting HSV from the eye to the CNS.

Virulence. In the first study (table 6, experiment

Table 5. Protection of guinea pigs against intravaginal challenge with 10⁵ pfu of HSV-2(G).

Test measurement	No. positive/total no. tested after immunization					
	Intradermal		Intramuscular			
	Control	HSV-2(G) (10 ⁷ pfu)	R7017 (10 ⁴ pfu)	R7020 (10 ⁴ pfu)	R7017 (10 ⁴ pfu)	R7020 (10 ⁴ pfu)
Vaginal shedding	10/10	1/5	7/10	9/10	10/10	10/10
Genital lesions	9/10	1/5	7/10	7/10	9/10	7/10
Paralysis	7/10	0/5	0/10	0/10	0/10	1/10
Death	5/10	0/5	0/10	0/10	0/10	0/10
Latency*						
Total	5/5	1/5	3/10	2/10	6/10	2/10
L3-L6	2/5		0/10	0/10	2/10	2/10
SI-S3	4/5		3/10	2/10	5/10	1/10

* L, lumbar; S, sacral.

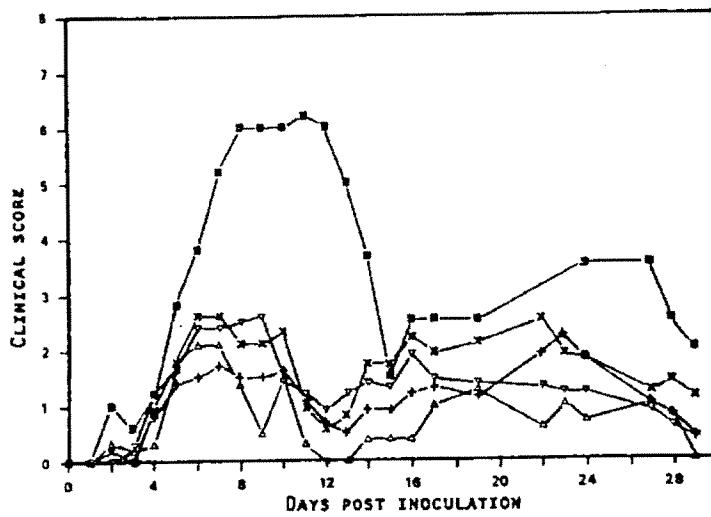


Figure 4. Score of perineal lesions after virulent challenge in control and immunized guinea pigs. Lesions were individually recorded by using a two-digit score modified by Stanberry et al. [26]. A first number was used to record the following progression of the lesions: 1, redness; 2, vesicle; 3, pustule; 4, ulcer; and 5, necrosis and loss of substance. This number was also used during the healing phase to record the following regression: 4, moist, soft, yellow crusts; 3, dry, dark scabs, still with inflammatory halo; 2, same but without inflammation; and 1, thin, pinkish, new epithelium. A second number was used to represent the extent of the lesions: 1, one or few well-individualized lesions; 2, confluent lesions, usually covering a large area; and 3, lesions covering the entire perineum. The numbers on the ordinate represent the daily score of each group, i.e., the mean of the individual sums of the two scores in all animals, symptomatic or not. ■, Scores for control animals; +, R7017 given intradermally; x, R7017 given im; △, R7020 given intradermally; and ▽, R7020 given im.

1), we found that HSV-1(F) can cause symptomatic infections following inoculation of 50 or 100 μ L containing 10^7 pfu on the right eye after scarification of the cornea with eight superficial strokes with a hypodermic needle. All four rabbits developed a

characteristic herpetic eye disease, survived, and harbored the virus in latent form. In the first series of experiments with the recombinant virus (table 6, experiment 2), groups of six rabbits were inoculated as above but with 10^4 pfu. The rabbits were then

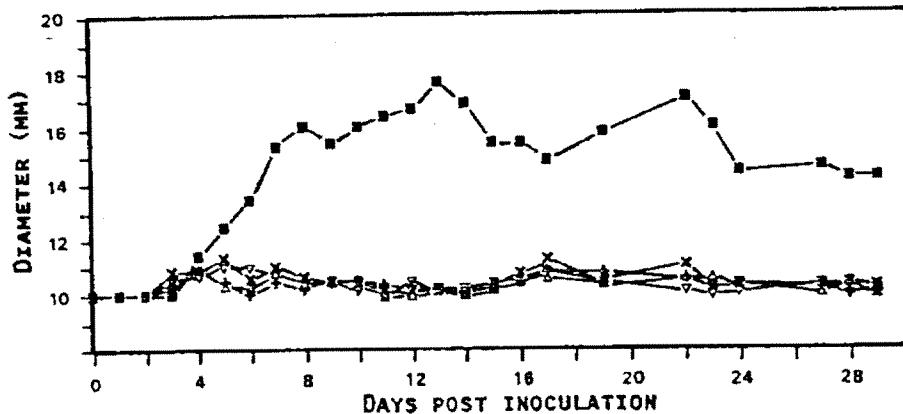


Figure 5. Perineal tumefaction after virulent challenge in control and immunized guinea pigs. The size of the glabrous area surrounding the vulva was determined by averaging its vertical and horizontal diameters for each animal. The numbers on the ordinate represent the mean value calculated daily for each group. The symbols are the same as those in figure 4.

Table 6. Outcome of ocular infection with HSV in rabbits.

Test measurement	No. positive/total no. tested after inoculation with			
	HSV-1(F) (10 ⁴ pfu)	R7017 (10 ⁴ pfu)	R7020 (10 ⁴ pfu)	
Exp 1	Exp 2	Exp 3	Exp 2	
Shedding of virus	4/4	6/6	11/12	6/6
Symptoms				
Ocular	4/4	6/6	10/12	6/6
CNS	0/4	0/6	0/12	0/6
Death	0/4	0/6	0/12	0/6
Latency	3/3	2/6	1/12	1/6

NOTE. Exp = experiment no.

monitored daily for ocular lesions and CNS symptoms and were swabbed on days 1, 2, 3, 5, and 7 to search for shedding of virus. Approximately four weeks after inoculation, the rabbits were tested for the presence of latent virus. In this instance (table 6), the virus was recovered for approximately three days from tear films of rabbits inoculated with R7017 and for as long as seven days in half of the rabbits inoculated with R7020. The symptoms produced in the rabbits were, as a rule, milder than those caused by the wild-type virus and were generally confined to blepharitis and conjunctivitis (figure 6). Pannus

or deep ulcers were not seen, and the rabbits exhibited no CNS symptoms. The recovery of latent R7017 virus from the trigeminal ganglia of two rabbits was particularly surprising in light of the fact that R7017 contains a deletion in the *tk* gene and that previous studies had indicated that such viruses do not establish latency [28]. To verify this observation, we repeated the experiment with R7017. In this instance (experiment 3), 24 rabbits were inoculated as above. At daily intervals during the acute phase of the infection (days 2, 4, and 7), four rabbits were killed and tested for the presence of replicating virus in the trigeminal ganglion; the remaining 12 rabbits were tested for latent virus approximately four weeks after inoculation. We were unable to demonstrate the presence of replicating virus during the acute phase of the infection, but one of the 12 rabbits tested for latency yielded virus from the trigeminal ganglion.

Protection of rabbits against challenge with HSV-1(McKrae) by the eye route. In this series of experiments, rabbits were immunized im in the right thigh with 10⁷ pfu of R7017 or R7020 (in 1 mL). Approximately four weeks after mock immunization or immunization, the rabbits were challenged with 10⁶ pfu of the HSV-1(McKrae) strain. Challenge of control-immunized rabbits caused a typical herpetic disease in the inoculated eye of all animals and death in three

Figure 6. Score of ocular lesions in rabbits inoculated with wild-type HSV-1(F) (●) or with recombinants R7017 (+) or R7020 (○). Lesions were individually recorded by summing the numbers assigned to each clinical manifestation as follows: on the eyelids—lacrimation or purulent matter (1), redness (2), swelling (3), inflammation (4), and stenosis or ectropion (5); for the episcleral blood vessels—diffuse redness (1), discrete hyperemia (2), vessels circling iris (3), partial pannus (4), and total pannus (5); on the cornea—dull (1), fogged (2), or opaque (3). The daily clinical score for each group, plotted on the ordinate, was calculated as the mean of the individual values. The results shown for the recombinants were obtained in experiment 2 with 10⁴ pfu (see text and table 6). The experiment with HSV-1(F) was done separately with 10⁴ pfu, and the course of the lesions is shown for comparison.

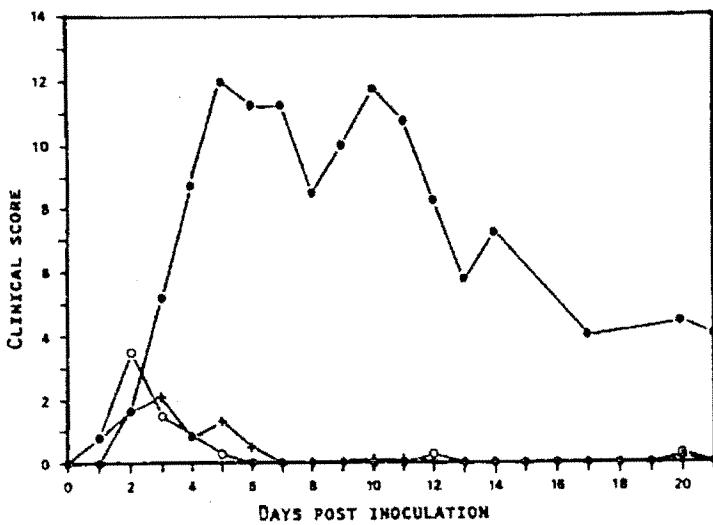


Table 7. Protection of rabbits against infection due to HSV-1 (McKrae) by im immunization with 10^7 pfu of R7017 or R7020.

Test measurement	No. positive/total no. tested with immunizing virus		
	Control	R7017	R7020
Shedding of virus	5/5	5/5	5/5
Symptoms			
Ocular	5/5	5/5	5/5
CNS	2/5	0/5	0/5
Death	3/5	0/5	0/5
Establishment of latency	1/2	1/5	0/5

of five animals (table 7). Previous immunization with the recombinant viruses protected against CNS symptoms and death but not against clinical infections of the eye. The signs of conjunctivitis, however, disappeared faster in immunized animals (days 12-14, compared with days 20-24 in control animals), and later on, milder forms of keratitis were observed among the "vaccinees."

Discussion

In this paper we report the biologic properties of two recombinant viruses in mice, guinea pigs, and rabbits. The salient feature of these viruses is that they have a grossly reduced pathogenicity and ability to remain latent in sensory ganglia of these hosts. Immunization with recombinant viruses protected against death and severe symptoms of disease after challenge with wild-type viruses. The recombinant viruses did not protect against infection with wild-type viruses. Several of these features merit further discussion.

The design of these recombinant viruses was based on two assumptions. The first was that deletion mutants, unlike mutants derived by base substitutions, are likely to be stable and would not acquire virulence by reversion or compensatory mutations elsewhere in their genomes. This expectation was fully borne out in previous studies [24], in which deletion and base-substitution mutants were compared by serial passage through mouse brain, as well as in this study, in which the R7017 and R7020 mutants were compared by serial passage.

The second assumption was based on the reports that *tk*⁺ viruses are more virulent and tend to establish latency in sensory ganglia than do those viruses lacking the enzymatic activity [28]. On the basis of

these observations, we expected that R7017 would be more attenuated than R7020 in the systems we tested. A comparison of R7017 and R7020 does not reveal a significant difference with respect to their ability to cause death in mice (pfu:LD₅₀) or disease patterns in guinea pigs and rabbits. Although we have not been able to detect latent R7017 virus in mice or guinea pigs, the incidence of latent R7020 was small. The fact that R7017 was able to establish latency in rabbits confirms that *tk*⁺ mutants are inherently able to establish latency in at least some of their hosts [29-31]. This does not appear to be a unique property of R7017 virus, because another virus, HSV-1(F) Δ 305, also established latency in the rabbit, nor is this phenomenon characteristic of the rabbit alone, because latent R7017 virus was also recovered from the lumbosacral dorsal root ganglia of vaginally infected marmosets (authors' unpublished data).

A key feature of both R7017 and R7020 is that the lesions caused by these viruses were localized, superficial, and healed much more rapidly than those caused by wild-type virus. We have not seen either the ulcerative lesions or the symptoms of CNS involvement that usually accompany HSV infections of rabbit cornea or of the genital tract of guinea pigs.

The ability of R7017 and R7020 to protect mice, guinea pigs, and rabbits from infection with wild-type viruses appears to be nearly equal, although in most of the tests done so far, R7020 appears to be slightly more protective. The protective potency of the recombinant viruses is lower, quantitatively, than that of wild-type virus; for example, in mice, nearly 100-fold more recombinant virus was needed to achieve the same level of protection as that obtained by immunization with wild-type viruses. When appropriate adjustments of immunizing doses of virus are made, however, the level of protection attained with wild-type and attenuated viruses was nearly equivalent: neither fully protects the immunized animal against replication or against the establishment of latent virus in sensory ganglia that enervate the site of inoculation of the challenge virus.

In the rodent species tested, a similar level of protection against challenge viruses was also attained after immunization with HSV glycoproteins, singly [32] or in artificial mixtures [33, 34]. We should note, however, that whereas this level of protection required more than one dose of subunit vaccine together with adjuvants, a single immunization with live virus was sufficient to protect the experimental

animals tested in this study. Furthermore, in our experience, subunit vaccines were effective against challenge viruses in rodent species but not in subhuman primates [33].

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EXHIBIT G

In Vivo Behavior of Genetically Engineered Herpes Simplex Viruses R7017 and R7020. II. Studies in Immunocompetent and Immunosuppressed Owl Monkeys (*Aotus trivirgatus*)

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The genetically engineered herpes simplex virus strains R7017 and R7020 were tested in owl monkeys (*Aotus trivirgatus*) previously shown to model herpetic diseases of immunocompromised patients and neonates. In contrast to the lethal disease seen in monkeys receiving 100–1,000 plaque-forming units (pfu) of wild-type virus, inoculation of $\geq 10^6$ pfu of recombinant viruses produced local lesions and viral shedding but not disseminated disease. Latent recombinant viruses were recovered from some ganglia innervating the sites of inoculation. Monkeys protected from lethal infection with wild-type virus exhibit recurrent lesions that increase in frequency and severity after total lymphoid γ irradiation (TLI). In contrast, monkeys immunosuppressed by TLI and inoculated with R7020 could not be differentiated from irradiated controls with respect to morbidity or mortality. Moreover, the virus was not transmitted from immunosuppressed infected females to normal male cage mates.

We reported earlier the construction of two genetically engineered herpes simplex (HSV) recombinant viruses, R7017 and R7020, and their behavior in rodent models [1]. These viruses were designed as prototypes of live, attenuated, genetically engineered vaccines against human herpetic infections. In rodent models the two constructs had considerably reduced virulence and ability to establish latency and could induce protective immunity. The recombinants did not change DNA restriction enzyme cleavage patterns or become virulent when subjected to selective pressure achieved by nine serial passages in the mouse brain. The need to evaluate the safety of these viruses in animal models that would mimic more closely the severe forms of herpetic diseases seen in infants or in immunocompromised patients prompted further studies in *Aotus trivirgatus* (the owl monkey), previously shown to be extremely susceptible to HSV [2, 3].

We report here that although 100 plaque-forming units (pfu) of wild-type viruses administered by peripheral routes were fatal to owl monkeys, recombinants in quantities at least 10⁵-fold greater inoculated by various routes were innocuous or produced mild infections. When tested in parallel, the *tk*⁺ virus R7017 behaved similarly to the *tk*⁺ virus R7020, corroborating the results of studies in rodents. Immunosuppression by total lymphoid γ irradiation (TLI) did not significantly alter the tolerance to R7020 when applied before inoculation and did not cause clinical reactivation when applied to latently infected animals.

Materials and Methods

Cells and viruses. The recombinant R7017 and R7020 and wild-type HSV-1(MGH10) and HSV-2(G) viruses used in this study and the procedures for virus growth, virus isolation, and restriction endonuclease analyses of viral DNAs were as described elsewhere [1]. The R7017 recombinant consists of the HSV-1(F) genome from which ~500 base pairs (bp) of the domain of the *tk* gene, the two genes mapping between the $\alpha 27$ gene and the internal inverted repeats, and all of the internal inverted repeats up to the EcoRI site in the $\alpha 4$ gene had been removed. In place of the internal inverted repeats were inserted a DNA fragment encoding the HSV-2(G) glycoproteins G, D, and I and a truncated part of glycoprotein E. To construct R7020, a DNA fragment containing the 5' promoter of the $\alpha 4$ gene fused to the transcribed domain of the *tk* gene was inserted between the HSV-2 fragment and residual part of internal inverted repeats of the R7017 virus.

Immunoprecipitation tests. Immunoprecipitation tests were done as described by Pereira et al. [4].

Primate studies. Quarantined wild-caught *A. trivirgatus* monkeys, seronegative for HSV, were purchased from Charles River Research Primate Corp. (Port Washington, NY) or from the Pan American Health Organization (Washington, DC) and transferred to France in compliance with national and international regulations. Except as stated in the text, they were housed in individual cages without physical contact with each other. The monkeys were allowed to adapt to the facility for a minimum of 4 months before the first inoculations.

Inoculations, venipunctures, and irradiations were done under ketamine hydrochloride anesthesia (8 mg/kg). Euthanasia was obtained with pentobarbitone overdose (30 mg/kg) in ketamine hydrochloride-anesthetized animals. Intravaginal inoculations were done by introducing 100–600 μ l (to make up for the differences in infectious titer of the virus stocks) of virus suspension after swabbing to remove genital secretions; in some instances, small cotton or gelatin pellets were introduced into the vagina to prevent leakage of the inoculum. Ocular inoculations were done by instilling 50 μ l of virus

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suspension in the right eye without scarification of the cornea; the eyelids were then gently closed and opened until the fluid was resorbed.

Physical examination of the monkeys included evaluation of general condition, reactivity, feeding, rectal temperature, skin, and mucosae. White and red blood cell counts (WBC and RBC) were determined with a Coulter counter according to the manufacturer's instructions (Coultronics, Margency, France). Differential leukocyte counts were done on coded blood smears stained with May-Grünwald-Giemsa.

TIL. The protocol was derived from that described by Lowder et al. [5]. Briefly, monkeys were exposed to doses of 50–300 rad three or four times a week from a cesium-137 source (Irradiator Lisa, Unilabo, Levallois-Perret, France). The skull, chest, and right lobe of the liver were protected with a lead shield. WBC were monitored to adjust the irradiation doses to maintain severe leukopenia (~ 2000 cells/mm 3) compatible with survival for several weeks.

Statistical analyses. Statistical analyses were done by the χ^2 test, with the Yates's correction when appropriate.

Results

Inoculation of Recombinants R7017 and R7020 in Immunocompetent Monkeys

Pathogenicity in owl monkeys. Three series of experiments were done. In the first, owl monkeys were inoculated with wild-type strain HSV-1(MGH10) or HSV-2(G) or with recombinant virus R7017 or R7020 at various dosages and routes (table 1). In the second series (table 2), two groups of six monkeys were infected with each recombinant virus by multiple routes simultaneously, that is, with 10^6 pfu by the ocular route and 10^7 pfu by vaginal and subcutaneous routes. In the last series of experiments, three monkeys were injected intravenously with 10^7 pfu of R7020.

In the first two experiments (tables 1, 2) infections of monkeys with ≥ 100 pfu of HSV-1(MGH10) or HSV-2(G) by either vaginal or intradermal routes were invariably fatal. Monkeys inoculated by the vaginal route developed hyperthermia a few days after inoculation and became hypothermic and lethargic shortly before death, 7–12 days after inoculation. They showed extensive ulcerative lesions of the perineum, often worsened by scratching. Virus was readily isolated from saliva, tear film, and vaginal secretions and from most tissue specimens tested after necropsy. Intradermal inoculation in the foot caused minimal inflammatory or localized vascular lesions but produced systemic disease with death in 10–16 days.

In contrast, all monkeys survived infection with either R7017 or R7020 at dosages 10^4 – 10^5 times higher. As a general rule, monkeys exhibited either no or minimal lesions at the site of inoculation and remained without symptoms throughout the observation period. Body temperatures remained within physiologic limits; in two instances, hyperthermia ~ 2 weeks after infection resolved with kanamycin therapy (Kamycine, Laboratoires Bristol, Paris; 50 mg/kg/day). The condition and

local reactions of the monkeys infected with the recombinant viruses by multiple routes did not differ from those of monkeys inoculated by a single route.

Eighteen monkeys were inoculated by the vaginal route (tables 1, 2). These included 8 with R7017 (nos. 218, 220, and 401–406) and 10 with R7020 (nos. 214, 216, 217, 219, and 407–412). Three exhibited no or minimal symptoms, although virus was recovered for a minimum of 6 days from vaginal secretions of all animals. The other monkeys developed single vesicular lesions and moderate swelling of the vulva. Between postinfection days 11 and 16, one female (no. 407) showed marked inflammation and localized necrosis of the vulvar margin, which healed after treatment with kanamycin (described above) and antibiotic ointment (Pommade Triantibiotique Monot, Quétigny, France). The monkeys never exhibited purulent discharge or evidence of pruritus. The lesions improved in a few days and completely disappeared in the time interval indicated in tables 1 and 2. In the monkeys infected only by the vaginal route (nos. 218, 220, 216, and 219), virus isolations from other sites were sporadic.

The monkeys inoculated by the ocular route with R7017 or R7020 developed conjunctivitis and ≤ 15 small, stellar corneal ulcers that disappeared within 3 weeks without sequelae or recurrences. Some monkeys also developed signs of uveitis of variable severity. Virus was isolated from the tear film of all but two monkeys, from day 2 up to day 30 in the extreme case. The control eye remained free of lesions for the duration of the study.

The monkeys inoculated intramuscularly showed only muscular stiffness for 1 or 2 days after injection; no cutaneous lesions were noted. Subcutaneous immunization yielded either no lesion or single small erythematous plaques (< 5 mm in diameter) at the point of skin puncture; no vesicular or pustular lesions were noted (tables 1, 2).

In the third experiment, recombinant R7020 was cleared rapidly from the bloodstream after intravenous injection. Viremia was detected (~ 100 pfu/ml) in two of three monkeys (nos. 506 and 507) at 2 h after infection. No virus was recovered from further blood specimens taken at 5, 9, and 24 h after injection, but virus was isolated from the saliva of the same monkeys on days 5 and 14 after infection. All three monkeys lost weight ($\sim 10\%$); two were apparently healthy, and the third (no. 506) was febrile on days 4–8 but without specific sign of herpetic illness.

Latency. The results of the assays for latent virus in owl monkeys tested 6–7 weeks after intravaginal infection (nos. 218, 220, 216, and 219) are summarized in table 1. Latent virus was detected only in the dorsal root ganglia (DRG) innervating the site of inoculation. Infectious virus was not detected in any of the ganglia tested. Of the two monkeys injected subcutaneously with R7020, one failed to yield latent virus and the other harbored latent virus in one thoracic and three lumbar DRG. Of the three monkeys injected intravenously,

Table 1. Summary of studies of wild-type and recombinant virus infections of *Aotus trivirgatus* after a single peripheral inoculation.

Virus, monkey no.	Inoculum route, pfu	Duration (days after infection)	Specimens positive for virus			
			Shedding	Local symptoms	Infectious	
					Tissues (n = 6)	DRG
HSV-1(MGH10)						
750	ID, 10 ²	6-14	-	15	3	
760	ID, 10 ²	12-19	-	19	1	
717	V, 10 ²	2-8	6-9	9	6	
729	V, 10 ²	2-8	6-9	9	5	
HSV-2(G)						
716	ID, 10 ²	4-8	8-10	10	6	
722	ID, 10 ²	10-12	-	12	6	
754	V, 10 ²	2-4	3-4	4*	2	
799	V, 10 ²	2-6	5-7	7	6	
103	V, 10 ³ †	2-6	3-6	6	6	
106	V, 10 ³ †	2-8	3-8	8	5	
107	V, 10 ³ †	2-8	4-9	9	6	
212	V, 10 ³ †	2-8	4-8	8	6	
213	V, 10 ³ †	2-6	4-8	8	6	
R7017						
218	V, 10 ⁷	2-14	2-23	S	0	0/36
220	V, 10 ⁷	2-6	-	S	0	0/38
201	O, 10 ⁶	2-20	5-20	S	ND‡	
202	O, 10 ⁶	2-14	4-19	S	ND‡	
R7020						
216	V, 10 ⁷	2-8	2-5	S	0	0/38
219	V, 10 ⁷	2-6	8-16	S	0	0/37
214	V, 10 ⁷	2-8	3-6	S	ND‡	
217	V, 10 ⁷	2-8	2-11	S	ND‡	
203	O, 10 ⁶	2-14	3-17	S	ND‡	
204	O, 10 ⁶	2-12	4-19	S	ND‡	
205	IM, 10 ⁷ **	-	-	S	ND‡	
215	IM, 10 ⁷ **	-	-	S	ND‡	
505	SC, 10 ⁷	-	-	S	ND	4/37††
510	SC, 10 ⁷	-	-	S	ND	0/37

NOTE. DRG = dorsal root ganglia, ID = intradermal, V = vaginal, O = ocular, IM = intramuscular, SC = subcutaneous, S = survived, ND = not done, - = none. Tissues examined were brain, liver, lung, kidney, adrenal gland, and cardiac blood. DRG were examined only on monkeys indicated; in each monkey of 200 series, left DRG were examined for infectious virus and right DRG were examined for latent virus. Sensory ganglia were paired and examined for latent virus only in monkeys 505 and 510. These monkeys were immunized 3 months after irradiation studies reported in table 6; at that time, WBC were back to baseline level.

* Accidental death.

† Control monkeys in protection studies reported elsewhere [6].

‡ Monkeys were used in challenge studies reported elsewhere [7].

§ DRGs S2 and L4.

¶ DRGs S2 and S3.

** Inoculated twice, 11 weeks apart.

†† DRGs T12, L1 to L3.

one (no. 507) was negative, and <10 pfu were recovered from T9 and S3 DRG of monkey 506 and from T13, L1, and L6 from monkey 508.

Spontaneous reactivation. The 12 owl monkeys listed in table 2 were monitored for spontaneous reactivation for 7 months beginning 1 month after inoculation by daily clinical examination and twice weekly by cultivation of vaginal secretions and tear film from the inoculated eye (~1,400 specimens). Although no lesions were detected, virus was isolated

from vaginal secretions on six occasions from 4 monkeys. While 6 monkeys were immunosuppressed (described below), the other 6 were monitored for another 8 months. Virus was isolated from only 1 of the latter monkeys (tear film of 408) of 880 specimens cultured (table 2). Latent R7020 virus was detected in the DRG even in 2 monkeys that failed to reactivate during the 16-month follow-up period.

The DNAs of the isolates from the DRG reported in table 1 and of the isolates from spontaneous reactivations reported

Table 2. Summary of studies with recombinant virus infections of *Aotus trivirgatus* after multiple peripheral inoculations.

Virus, monkey no.	Last day of shedding	Duration of lesions (days after infection)			Spontaneous reactivation (days after infection)	Latent virus in sensory ganglia*
		Cornea	Subcutaneous site	Vulva		
R7017						
401	6	—	8-12	5-13		1/33†
402	16	4-20	7-14	5-12	112	0/36†
403	10	4	—	5-11		1/36†
404	6	—	7-13	2-11		NT
405	20	4-14	2	2-10		NT
406	24	4-20	7-11	5-8		NT
R7020						
407	12	4	7-8	5-25		2/36
408	26	4-24	8-9	4-10	331	2/37
409	30	4-26	6-8	4-8	225	1/35†
410	6	—	8-9	5-8		2/38
411	26	4-26	8-13	4-12	83, 106, 111	0/36‡‡
412	24	22-24	—	4-10	163	2/33†

NOTE. Virus administered at 10^6 pfu by ocular route, 10^7 pfu by vaginal route, and 10^7 pfu by subcutaneous route. Starting 1 month after infection, monkeys 401-403, 409, 411, and 412 were monitored for 8 months and monkeys 404-408 were monitored for 16 months; swabblings of tear film and vaginal secretions were cultured twice weekly. NT = not tested.

* Including trigeminal and right series of dorsal root ganglia (DRG).

† Immunosuppressed (see table 3).

‡ Of 15 left DRG assayed for infectious virus, 1 was positive.

in table 2 could not be differentiated from those of the parental stocks of R7017 and R7020 viruses with respect to restriction endonuclease patterns (data not shown).

TLI of Infected Monkeys

The purpose of these studies was to determine whether spontaneously reactivated virus could cause clinical lesions in immunosuppressed monkeys. Preliminary studies indicated that conventional procedures for immunosuppression (e.g., administration of azathioprine and prednisolone, cyclophosphamide, or cyclosporin) were not effective in owl monkeys (data not shown). We therefore turned to TLI, an effective immunosuppressive therapy (reviewed in [8]).

Immunosuppression of owl monkeys. The 12 owl monkeys selected for these studies consisted of four groups. The first two groups (the 200 series or A groups) had been inoculated with recombinants R7017 or R7020 and challenged with wild-type virus HSV-2(G) [7]. These monkeys developed herpetic infections that healed in a few weeks and spontaneous recurrent lesions from which only the challenge HSV-2(G) virus could be isolated. The second two groups (the 400 series or B groups) included 6 of the 12 monkeys inoculated simultaneously with the recombinants R7017 or R7020 by several routes (table 2). They were subjected to TLI after a period of observation to establish the pattern of virus recurrences and the background count of WBC. Table 3 lists the history of virus inoculations, the TLI regimens to which the animals

were exposed, and the differential WBC counts before and after administration of ≥ 700 rad (i.e., 1 week after beginning irradiation).

The biology of irradiated owl monkeys. Clinical disease was similar in groups A and B, although it evolved at different rates. Except for a darkening of the glabrous skin of the face, hands, and feet and of the perineum, the physical condition of the monkeys remained normal for 1-2 weeks. Later, asthenia, anorexia, and weight loss were not uncommon. Respiratory disorders and hyperthermia, resistant to antibiotic therapy, and petechiae, hematomas, and epistaxis were noted in several animals. Hypothermia and prostration developed in the last few days before death.

Irradiation had a profound effect on WBC (table 3), RBC, and platelet counts. All subpopulations of WBC decreased similarly; data on basophils and eosinophils are not shown because they represented only a small fraction of WBC (1% and 8%, respectively). Notwithstanding individual differences in the WBC counts before irradiation and in the response to the initial doses of irradiation, all monkeys reached a level 10%-20% of the initial levels within 1 week of treatment. Reducing further the number of WBC required a considerable increase in irradiation (data not shown). The RBC also were depleted; their count declined gradually to $\sim 2 \times 10^6/\text{mm}^3$ at the time of death, whereas normal counts were $\sim 5 \times 10^6/\text{mm}^3$. Platelets were not counted, but their progressive disappearance was noted on blood smears.

At necropsy, the most common histopathologic findings

Table 3. Summary of irradiation protocol of herpes simplex virus (HSV)-infected monkeys.

Group, monkey no.	Irradiation			Neutrophils/mm ³		Lymphocytes/mm ³	
	Post-infection interval (days)	Total (rad)	No. of exposures	Survival (days)	Before irradiation	During irradiation course	Before irradiation
Group A1, R7017/HSV-2(G)							
201	8	900	9	30	6440 ± 1009	1201 ± 667	6430 ± 1260
202	8	1750	15	31*	4639 ± 1075	1025 ± 453	4128 ± 231
Group A2, R7020/HSV-2(G)							
203	8	900	8	17	5671 ± 1175	3479 ± 2420	4177 ± 146
204	8	700	7	13	7772 ± 1144	909 ± 335	10252 ± 1851
205†	5	800	8	—	8745 ± 908	2290 ± 1774	5577 ± 1391
205‡	3	2250	15	31*	4805 ± 499	1177 ± 580	3418 ± 322
217	6	900	9	42	10855 ± 1995	1995 ± 498	11069 ± 2747
Group B1, R7017							
401	8	3700	22	39	1484 ± 616	191 ± 105	6160 ± 491
402	8	3700	22	39	2241 ± 97	638 ± 312	8001 ± 748
403	8	3700	20	39	3090 ± 856	277 ± 214	5177 ± 588
Group B2, R7020							
409	8	3300	20	38	2857 ± 372	663 ± 397	10051 ± 542
411	8	4000	23	39	2319 ± 159	486 ± 230	6603 ± 482
412	8	3750	22	40	4652 ± 765	414 ± 423	8779 ± 552

NOTE. Neutrophil and lymphocyte counts are number ± SD. Monkeys were exposed to radiation until death except for first course (16 days) for 205 and 217. Monkeys in Groups A1 and A2 were challenged with 10³ pfu of HSV-2(G) intradermally 4–5 months after first virus inoculation [7]. Group B1 and B2 monkeys were infected with virus by ocular (10⁶ pfu), subcutaneous (10⁷ pfu), and vaginal (10⁷ pfu) routes.

* Sacrificed when moribund.

† Irradiated for 12 days in first experiment, then allowed to recover for 3 months before second course of irradiation.

‡ Second course of irradiation.

were hemorrhagic foci in myocardium, kidneys, lungs, or liver. Liver degeneration was the second most frequent observation. Occasional ascites or splenomegaly was also noted.

The effect of TLI on recurrent HSV lesions. We defined recurrent lesions as skin manifestations suggestive of HSV recrudescences and occurring >30 days after virus inoculation. Spontaneous recurrent lesions took four forms. Erosions ranged from small, superficial cracks to ulcers; they could be open or covered with a scab. Punctate lesions developed on intact skin and were flat, small, round inflammatory spots, ranging from pinpoint size to 1–2 mm in diameter. Vesicles and pustules were roofed lesions on an inflammatory halo with a serous or purulent content, respectively; these lesions ranged up to several millimeters. Lesions were single or clustered. In monkeys infected with wild-type virus (A groups), 159 such lesions were recorded before irradiation, 114 of which were scraped or swabbed for virus isolation, yielding 74 isolates. All four types of lesions yielded virus. The frequency of recurrences is shown in table 4. In most monkeys there was a trend to some periodicity, with clusters of recrudescences alternating with remission intervals (data not shown).

In monkeys infected with HSV-2(G), the frequency of recurrences increased 3- to 15-fold on irradiation (table 4), a statistically significant factor. Irradiation also increased the severity of the cutaneous recurrences; thus, the lesions developing during the irradiation period took at least 1 week to heal com-

pared with 1–3 days before TLI. In some cases, the lesions turned into ulcers surrounded with edema and associated with adenitis. Their propensity to expand led us to treat monkeys 201 and 217 with oral acyclovir, and rapid improvement ensued. Irradiation increased significantly the frequency of virus isolations from the recurrent lesions and in periodic cultures made of body fluids (table 5); analyses of the DNAs of 37 of 82 isolates from the monkeys in groups A indicated that they were all HSV-2(G).

In contrast, there was no significant change in the number of recurrent lesions in monkeys harboring only the recombinant viruses (B groups). However, the number of virus isolations from periodic cultures increased significantly in monkeys harboring R7020 (table 5). With one exception (a swollen vulva in monkey 411), shedding episodes were asymptomatic.

Recovery of virus from sensory ganglia after death. The left sensory ganglia were tested for infectious virus, and the right ganglia were tested for latent virus. Of significance, a large number of sensory ganglia from monkeys infected with HSV-2(G) yielded virus; a notable fraction of these ganglia contained infectious virus (table 5). In contrast, few ganglia taken from monkeys infected with the recombinant viruses yielded virus, and only one ganglion yielded infectious virus. The infected ganglia were those innervating the genital organs (i.e., only one of the three sites of simultaneous in-

Table 4. Effect of γ irradiation on clinical reactivation of herpes simplex virus strain G.

Monkey no.	Observation period		Preirradiation period		Irradiation period	
	Follow-up period (days)	Incidence of lesions	Follow-up period (days)	Incidence of lesions	Follow-up period (days)	Incidence of lesions
201	168	0.20	22	0.04	30*	0.87
202	170	0.14	17	0.29	31	0.77
203	148	0.03	21	0.00	17	0.47
204	138	0.19	17	0.12	13	1.92
205†	109	0.13			23	1.35‡
205†			23	0.17	31	0.39
217	149	0.39			23	1.17§

NOTE. Observation period began 30 days after HSV-2(G) inoculation. Preirradiation period was interval just before irradiation and did not overlap with observation period. Daily incidence of lesions was calculated by dividing number of clinical recurrent lesions during follow-up by number of days.

* Received oral acyclovir on days 18–30 (5 mg/kg/day).

† Received two courses of irradiation 3 months apart.

‡ Course of irradiation followed by 40 days without recurrent lesions.

§ Received oral acyclovir on days 24–28 (5 mg/kg/day).

oculation with the recombinant viruses). Thus, no virus was recovered from the ganglia innervating the subcutaneous and conjunctival sites of inoculation.

Inoculation of Recombinant R7020 in Irradiated Monkeys

We evaluated the tolerance of immunodepressed monkeys to immunization with recombinant R7020. Six naive monkeys were subjected to the irradiation protocol described above

for 22–29 days, receiving a total of 1350–1950 rad. On day 10, three female monkeys (nos. 501, 503, and 504) were injected with 10⁷ pfu of R7020 subcutaneously in the right thigh. Monkeys 505, 509, and 510, one female and two males, were uninfected controls.

Hematologic and clinical evolution under γ irradiation. The kinetics of the WBC and RBC counts in irradiated animals were similar to those seen in previous experiments. At the time of inoculation with R7020, the leukocyte counts were

Table 5. Effect of γ irradiation on recovery of reactivated virus.

Monkey no.	No. of virus isolations/no. of cultures from					
	Periodic specimens		Cutaneous recurrent lesions		Sensory ganglia	
	Before irradiation	During irradiation course	Before irradiation	During irradiation course	Infectious virus*	Latent virus†
Group A1, R7017/HSV-2(G)						
201	0/15	4/42	19/25	20/25	11/11	30/36
202	0/24	1/42	12/20	18/20	7/12	23/35
Group A2, R7020/HSV-2(G)						
203	0/21	5/24	3/3	7/8	4/11	7/23‡
204	0/18	3/21	16/22	20/20	7/12	13/35
205§	1/15	12/54	12/13	20/20	—	—
205§	3/18	3/42	8/8	10/12	2/12	25/36
217	0/51	10/51	12/31	14/16	3/12	15/36
Group B1, R7017						
401	0/116	0/36	0/1	0/1	0/13	1/33
402	1/116	0/36	NRL	0/1	0/14	0/36
403	0/116	1/36	NRL	NRL	0/14	1/36
Group B2, R7020						
409	1/116	2/36	NRL	NRL	0/14	1/35
411	3/116	12/36	NRL	NRL	1/15	0/36
412	1/116	3/36	NRL	0/1	0/13	2/33

NOTE. History of exposure of monkeys to wild-type and recombinant viruses is given in table 3; periodic specimens were tear film from right and left eyes and saliva in monkeys 201–205; right tear film, saliva, and vaginal secretions in 217; and right tear film and vaginal secretions in 401–412. Specimens were collected and tested twice weekly before and three times weekly during irradiation. NRL = no recurrent lesion.

* In every third left dorsal root ganglion (DRG) for 200 series and in left trigeminal and lumbar, sacral, and caudal DRG for 400 series.

† In right trigeminal ganglion and all right DRGs.

‡ Of 36 cultures, 13 were discarded because of bacterial contamination.

§ Two courses of irradiation of monkey 205.

~15% of initial levels and, in most animals, low counts were maintained for ~3 weeks after infection (table 6). WBC counts evolved similarly in inoculated and irradiated control monkeys (data not shown).

Four of the six monkeys showed symptoms as described above and survived the test. Monkey 501 showed a first episode of hypothermia, which prompted us to use oral acyclovir and supportive therapy, resulting in rapid but transitory improvement. This monkey and control monkey 509 then developed strikingly similar severe hypothermia and apathy, after 23 and 28 days of irradiation respectively. Supportive therapy, including glucose solution, vitamin C, terramycin (30 mg/kg/day) and one blood transfusion from unirradiated uninfected monkeys, had little effect on their condition and they were euthanized on days 49 and 37, respectively. At necropsy, both monkeys were heavily infested with oxyuris. Histopathology revealed no lesions suggestive of herpetic infection.

Monkey 503 showed no lesions at the site of subcutaneous injection. Monkey 501 had a papule (1-2 mm) on days 5-7 after injection and monkey 504 had a tiny pustule (2 mm) on an inflammatory base on days 7-9.

Isolation of the recombinant virus R7020 from immunized monkeys. For 130 days after injection, the inoculated monkeys were monitored three times weekly for shedding in the right-eye tear film, the saliva, and the vaginal secretions. Cultures were also made of skin swabbings at the site of injection until postinfection day 18. Of 370 specimens tested, only 1, from vaginal secretions of monkey 501 on day 11, yielded virus. No virus could be isolated from postmortem specimens of brain, lung, spleen, liver, kidney, adrenal glands, and heart blood of monkey 501.

Distribution of latent virus in sensory ganglia. Monkey 501 was examined for the presence of infectious virus in the right series of sensory ganglia and latent virus in the left series. While the former set of cultures was negative, latent virus was detected in a small amount (≤ 10 infectious foci) in DRGs T5, T6, T9, and T10. Monkeys 503 and 504 were tested only for latent virus on paired sensory ganglia. No virus was recovered from monkey 503, and isolates were obtained from DRGs T14, L2, and L3 of monkey 504.

Transmission of R7020 from Inoculated *A. trivirgatus* Monkeys to Sentinel Mates

To test the ability of recombinant R7020 to spread from the recipients to naive companions, three male monkeys were mated, as sentinel animals, with the immunocompromised inoculated females described in the previous section. Each couple (nos. 501 and 506, 503 and 507, and 504 and 508) was housed separately.

No males developed lesions or showed changes in weight or behavior during the 4-month observation period after inoculation of the females. No antibody was detected in eight serum specimens taken at weeks 2-7, 11, and 15 that were

Table 6. White blood cell counts in *Aotus trivirgatus* monkeys inoculated with R7020 while subjected to total lymphoid irradiation.

Monkey no., irradiated	Inoculated with R7020	Leukocytes/mm ³ $\times 10^{-3}$			Last irradiation
		Day 0	Day 10		
501, Yes	Yes	8.4	1.5	0.4*	
503, Yes	Yes	13.1	2.4	1.3†	
504, Yes	Yes	10.4	1.6	1.9†	
505, Yes	No	7.7	1.4	1.0†	
509, Yes	No	9.1	1.6	0.8‡	
510, Yes	No	12.1	1.3	1.6†	
506, No	No	19.3	19.1	18.7†	
507, No	No	11.9	14.6	14.7†	
508, No	No	5.2	5.7	4.0†	

NOTE. Monkeys were inoculated on day 10.

* Day 22.

† Day 29.

‡ Day 25.

examined by immune precipitation. In the same test, antibodies were detected in a 1:1000 dilution of the serum from monkey 407 taken 6 weeks after immunization.

Discussion

HSV pathogenesis in various animal model systems: the unique characteristics of the owl monkey model. Central to the biology of HSV is the difference in their dissemination in humans depending on age and immune status. In healthy individuals beyond the age of a few weeks, the infection may range from asymptomatic to severe but localizes to the portal of entry. Rarely is virus transported to the central nervous system, probably by neurotropic spread, or to the viscera [9]. In contrast, neonatal infection is almost invariably symptomatic and frequently disseminated [10], suggesting a lymphatic or bloodborne spread. In immunocompromised persons, exogenous or activated latent virus can spread from the site of infection to contiguous tissues, causing debilitating infections but usually without visceral dissemination [11-13].

A key requirement of a live virus vaccine is that it should be safe even in the most susceptible human populations. Animal models are necessary to safety testing, but no animal species exhibit in toto the range of human herpetic diseases. Further, there is a large diversity of susceptibility among species. Here we have addressed the issues of safety of the genetically engineered viruses R7017 and R7020 in a model that approximates closely the neonate and the immunocompromised person. Rodent models (e.g., mice and guinea pigs) and rabbits were not suitable; even though they can succumb from HSV administered by peripheral routes, it is usually from paralysis and death caused by the neural spread of the virus from the site of inoculation to the central nervous system, a situation significantly different from that seen in neonates. Also, recurrent lesions are uncommon in mice and rabbits; while the guinea pig exhibits spontaneous clinical recurrences

after genital inoculation, the lesions are frequently sterile in contrast to human infections.

Of the models described in the literature, only *A. trivirgatus* monkeys exposed to virus by natural routes of human infection exhibit most features of neonatal infections [2, 3]. We report that they also develop recurrent lesions similar to those seen in humans.

Comparative pathogenicity of wild-type and recombinant viruses in owl monkeys. Previous studies used unspecified amounts [3] or relatively high doses ($\geq 10^6$ 50% tissue-culture infective dose) of type 1 virus inoculated by the ocular route [2] or by an unstated route [14]. In our study, doses as low as 100 pfu of either wild-type HSV-1 or HSV-2 per animal by intradermal or vaginal route produced fatal systemic infection. The owl monkeys succumbed to infection 7-19 days after infection, and on necropsy, virus was isolated from most organs and body fluids. Hepatitis and adrenal necrosis rather than encephalitis were the hallmarks of the infection of *A. trivirgatus* monkeys.

In contrast to wild-type viruses, the two recombinant viruses in doses 10^4 - 10^5 times higher produced no significant disease. Thus, injections of 10^7 pfu of R7017 or R7020 into immunocompetent owl monkeys by intramuscular or subcutaneous routes produced no significant side effects. Inoculation with 10^6 pfu on the vaginal or ocular mucosa, peripheral routes that enable better monitoring of infection, showed that the recombinant viruses could be detected for up to 3 weeks. In no instance, however, were these infections fatal or harmful to the monkeys, and the most dramatic symptoms of infections were mild local inflammation.

Comparative ability of wild-type and recombinant viruses to establish latent infections in owl monkeys. Inasmuch as wild-type infection was fatal, the latency studies with wild-type viruses were done on animals immunized with R7017 or R7020 before infection with wild-type viruses; they showed that HSV-2(G) established latency and further that this virus was readily isolated from recurrent lesions. Because recombinant viruses were not lethal, they lent themselves to analyses of latency and reactivation.

Both R7017 and R7020 established latent infections. However, latent virus could be recovered from only the ganglia innervating the site of inoculation. The number of ganglia from which latent virus could be recovered after intravenous injection of 10^7 pfu of R7020 was also small. The efficiency of establishment of latent infections varied depending on the route of infection; most efficient was the vaginal route. In contrast to animals infected with HSV-2(G), reactivation of latent recombinant virus was rare and subclinical and would not have been detected without intensive monitoring.

We did not observe significant differences in the behavior of R7017 and R7020, and there seems to be no advantage to using *tk*⁻ virus as a vaccine. On the contrary, using *tk*⁺ virus is advantageous; in the event of viral spread, infection

is amenable to chemotherapy by drugs that require viral thymidine kinase for activation.

Recurrence and dissemination of viruses in and among susceptible animals. The key questions for which there was no test model system are the effect of immunosuppression in animals carrying the recombinant virus in latent form, the susceptibility of naive immunocompromised animals to infection with recombinant viruses, and the transmissibility of recombinant viruses by physical contact.

To test the effect of immunosuppression on the resident latent virus, monkeys infected by several routes were exposed to TLI. Monkeys carrying latent wild-type virus exhibited new and more severe recurrent lesions and shed virus more often than did immunocompetent animals. In contrast, although irradiation increased the recovery of virus from monkeys infected with recombinant viruses, reactivation episodes were asymptomatic and did not lead to the formation of lesions. A similar contrast was found in the latency studies: Although infectious wild-type virus was recovered from most sensory ganglia examined, recombinant virus was detected, mostly as latent virus, from few ganglia, and these generally innervated the immunization site.

The susceptibility of naive immunocompromised animals to recombinant viruses was tested by administering R7020 to animals with TLI-induced leukopenia. In this study, irradiated monkeys that received 10^7 pfu of R7020 by subcutaneous routes could not be differentiated from irradiated control animals with respect to disease or death.

To determine whether infected immunocompromised animals tended to transmit virus to naive animals by physical contact, irradiated females immunized subcutaneously with 10^7 pfu of R7020 were mated with naive males. Because the males remained seronegative, we conclude that virus administered subcutaneously was not available for dissemination by physical contact.

Evaluation of the safety of R7017 and R7020 as candidates for human vaccination. Of the known animal models of human HSV infections, owl monkeys are the most susceptible and represent the closest approximation of the most susceptible human population. In contrast to wild-type HSV, the recombinant viruses R7017 and R7020 do not appear to spread from the site of inoculation, their ability to cause disease is limited and independent of the route of inoculation, and their capacity to cause disease and to spread was not exacerbated by TLI. In this regard, the recombinant viruses meet the safety requirements expected of genetically engineered live viruses suitable for human use.

In these studies, we extend our previous findings [1] that R7020 and the *tk*⁻ recombinant R7017 can establish latent infection in various species. The significant observations are that (1) the efficiency of establishing latent infection depends on the route of inoculation, (2) no spontaneously reactivated virus could be detected after subcutaneous or intramuscular

inoculation, and (3) herpetic diseases were not seen on irradiation of latently infected owl monkeys that had shed spontaneously reactivated virus.

The role of latent virus in the pathology and immunity induced by HSV has been extensively discussed. In humans infected with wild-type virus, recurrent infections are a major cause of discomfort and disease. Nevertheless, there is evidence that prior infection with one virus can prevent or mitigate superinfection with another virus [9, 15]. The capacity of the recombinant viruses to establish latency must therefore be viewed in light of the potential prolongation of protection against new infections associated with the presence of latent virus.

The ideal live HSV vaccine would confer a high initial and long-lasting level of immunity, would not cause recurrent lesions, and would not be transmitted from person to person. In the highly susceptible *A. trivirgatus* monkey model, R7020 appears to meet the latter two criteria despite its ability to remain latent.

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